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PURINERGIC SIGNALING REGULATES FILOPODIA-INDUCED ZIPPERING

Jessica Lynn Bowser

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PURINERGIC SIGNALING REGULATES FILOPODIA-INDUCED ZIPPERING

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PURINERGIC SIGNALING REGULATES FILOPODIA-INDUCED ZIPPERING

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Jessica Lynn Bowser, M.S.

Houston, Texas

December, 2011

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PURINERGIC SIGNALING REGULATES FILOPODIA-INDUCED ZIPPERING

Publication No. _____

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The molecular mechanisms that mediate endometrial cancer invasion and metastasis remain poorly understood. This is a significant clinical problem, as there is no definitive cure for metastatic disease. The purinergic pathway's generation of adenosine and its activation of the adenosine receptor A2B (A2BR) induces cell-cell adhesion to promote barrier function. This barrier function is known to be important in maintaining homeostasis during hypoxia, trauma, and sepsis. Loss of this epithelial barrier function provides a considerable advantage for carcinoma progression, as loss of cell-cell adhesions supports proliferation, aberrant signaling, epithelial-to-mesenchymal transition, invasion, and metastasis. The present work provides strong evidence that CD73-generated adenosine actively promotes cell-cell adhesion in carcinoma cells by filopodia-induced zippering. Adenosine-generating ecto-enzyme, CD73, was down-regulated in moderately- and poorly-differentiated, invasive, and metastatic endometrial carcinomas. CD73 expression and enzyme activity in normal endometrium and endometrial carcinomas was significantly correlated to the epithelial phenotype. Barrier function in normal epithelial cells of the endometrium was dependent on stress-induced generation of adenosine by CD73 and adenosine's activation of A2BR. This same mechanism inhibited endometrial carcinoma cell migration and invasion. Finally, adenosine's activation of A2BR induced the formation of filopodia that promoted the re-forming of cell-cell adhesions in carcinoma cells. Overall, these studies identified purinergic pathway-induced filopodia to be a novel mechanism of adenosine's barrier function and a mechanism that has to be avoided/down-regulated by endometrial carcinoma cells attempting to lose attachment with their neighboring cells. These results provide insight into the molecular mechanisms of endometrial cancer invasion. In addition, because loss

of cell-cell adhesions has been closely linked to therapy resistance in cancer, these results provide a rational clinical strategy for the re-establishment of cell-cell adhesions to potentially increase therapeutic sensitivity. In contrast to other molecular mechanisms regulating cell-cell adhesions, the purinergic pathway is clinically druggable, with agonists and antagonists currently being tested in clinical trials of various diseases.

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LIST OF ABBREVIATIONS

18S rRNA	18s Ribosomal RNA
5'AMP	5' Adenosine Monophosphate
A1R	Adenosine A1 Receptor
A2AR	Adenosine 2A Receptor
A2BR	Adenosine 2B Receptor
A2BR ^{-/-}	Adenosine 2B Receptor Deficient
A3R	Adenosine A3 Receptor
ADA	Adenosine Deaminase
ADP	Adenosine Diphosphate
AK	Adenosine Kinase
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
AoPCP	α , β -Methylenediphosphate
aPKC	Atypical Protein Kinase C
AP-1	Activator Protein 1
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
Axin-GSK3 β -APC	Axin-Glycogen Synthase Kinase-3 β -Adenomatous Polyposis Coli
BAEC	Bovine Fetal Aortic Endothelial Cells
BSA/HCl	Bovine Serum Albumin/Hydrochloric Acid
C	Celsius
Ca ²⁺	Calcium
cAMP	cyclic Adenosine Monophosphate

C/EBP	CCAAT-enhancer Binding Protein
CD73	5'Nucleotidase
CD73 ^{-/-}	CD73 Deficient
CDK4	Cyclin Dependent Kinase 4
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CNTs	Concentrative Nucleoside Transporters
CO ₂	Carbon Dioxide
COX-2	Cyclooxygenase 2
Crumbs-PALS1-PATJ	Crumbs-Protein Associated with Lin Seven 1-PALS1-Associated TJ Protein
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
dH ₂ O	Distilled Water
Dlg	Discs Large
DMEM	Dubulcco Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EC	Endometrial Cancer
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
ENT1	Equilibrative Nucleoside Transporter 1

ENTs	Equilibrative Nucleoside Transporters
EtOH	Ethanol
FBS	Fetal Bovine Serum
FIGO	International Federation of Gynecology and Obstetrics
G1 EEC	Grade 1 Endometrial Endometrioid Endometrial Carcinoma
G2 EEC	Grade 2 Endometrial Endometrioid Endometrial Carcinoma
G3 EEC	Grade 3 Endometrial Endometrioid Endometrial Carcinoma
GPI	Glycosyl Phosphatidylinositol
H ₂ O	Water
HeLa	Henrietta Lacks
Her2/neu	Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HGPSC	High Grade Papillary Serous Carcinoma
HIF-1	Hypoxia-Inducible Factor-1
HIF-1 α	Hypoxia-Inducible Factor-1 α
HMEC-1	Human Microvascular Endothelial Cells
Hrs	Hours
HUVEC	Human Umbilical Vein Endothelial Cells
IGF1R	Insulin Growth Factor 1 Receptor
IGFBP1	Insulin-like Growth Factor-Binding Protein 1
IL-1 β	Interleukin-1 β
ILK	Integrin-Linked Kinase
INF- γ	Interferon- γ
IP	Intraperitoneal
IP3	Inositol Triphosphate

JAM1	Junctional Adhesion Molecule 1
JAM3	Junctional Adhesion Molecule 3
JAMs	Junctional Adhesion Molecules
kDa	Kilodalton
K_M	Michaelis-Menten Kinetics
Lgl	Lethal Giant Larvae
MAPK	Mitogen-Activated Protein Kinase
MDA-GCTR-TB	MD Anderson Multidisciplinary Gynecological Cancer Translational Research Tumor Bank
MEK	Mitogen Activated Protein Kinase Kinase
Mg^{2+}	Magnesium
MMMT	Malignant Mixed Müllerian Tumor
MMP-7	Matrix Metalloproteinase-7
$MnCl_2$	Manganese Chloride
MPA	Medroxyprogesterone Acetate
MRS1754	<i>N</i> -(4-Cyanphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1 <i>H</i> - purin-8-yl)phenoxy]-acetamide
mTOR	Mammalian Target of Rapamycin
N	Sample Number
N-Endo	Normal Endometrium
NECA	5'-N-ethylcarboxamidoadenosine
$(NH_4)_2S$	Ammonium Sulfide
NIH	National Institute of Health
NS	Non-Significant
NSCLC	Non-Small Cell Lung Cancer

O ₂	Oxygen
P2RY2	P2 Receptor, Purinergic Receptor P2Y
PALS	Protein Associated with Lin Seven 1
Par3	Partitioning-Defective Protein-3
Par6	Partitioning-Defective Protein-6
Par6-Par3-aPKC	Partitioning-Defective Protein-6- Partitioning-Defective Protein-3- Atypical Protein Kinase C
PATJ	PALS1-Associated TJ Protein
Pb(NO ₃) ₂	Lead(1) Nitrate
PBS	Phosphate Buffered Saline
PC12	Rat Adrenal Pheochromocytoma Cells
PCNA	Proliferating Cell Nuclear Antigen
PI-PLC	Phosphatidylinositol-Phospholipase C
PI3K	Phosphoinositide-3-Kinase
PI3K/Akt	Phosphoinositide-3-Kinase/Akt
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PMNs	Polymorphonuclear Leukocytes
PR β	Progesterone Receptor β
PTEN	Phosphatase and Tensin Homolog
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
rRNA	Ribosomal Ribonucleic Acid

RT-PCR	Reverse Transcription Polymerase Chain Reaction
Scribble-Dlg-Lgl	Scribble-Discs Large-Lethal Giant Larvae
SE	Standard Error
SIP1	ZEB2
siRNA	Small Interfering RNA
SNAI1	Snail
SNAI2	Slug
SSP1	Osteopontin
TCF/LEF1	T-Cell Factor/Enhancer-Binding Factor 1
TCR	CD3/T-Cell Receptor
TER	Transepithelial Resistance
TGF- β	Transforming Growth Factor- β
TGF- β 1	Transforming Growth Factor- β 1
TNF- α	Tumor Necrosis Factor- α
TSC2	Tuberous Sclerosis Complex 2
UPSC	Uterine Papillary Serous Carcinoma
VASP	Vasodilator-Stimulated Phosphoprotein
VDAC-1	Voltage-Dependent Anion Channel-1
VHL	von Hippel-Lindau
VSOR	Volume-Sensitive Outwardly Rectifying
WISH	Wistar Institute Susan Hayflick
WISH-HeLa	Wistar Institute Susan Hayflick-Henrietta Lacks
Zn ²⁺	Zinc
ZO-1	Zona Occludens 1
ZO-2	Zona Occludens 2

ZONABZO-1 associated nucleic acid binding protein
δEF1 ZEB1

LIST OF SYMBOLS

α	Alpha
β	Beta
$^{\circ}$	Degrees
δ	Delta
μ	Micro
%	Percent
®	Registered Trade Mark
™	Trade Mark

CHAPTER ONE

Prelude

Cancer remains a significant health concern as it is the second most common cause of death in the United States. Lifetime risk for developing cancer is slightly less than 50% for men; for women, the risk is a little more than 33% (1). Defined as a malignant growth caused by uncontrolled cell growth, cancer can arise in all tissue types. Cancers arising from transformation of epithelial cells (carcinomas) are the most common. In 2011, an estimated 1.5 million Americans are expected to be newly diagnosed with cancer. A mortality rate of 1,500 deaths per day is also expected (1). Though mortality rates for cancers arising in the breast, colon, and cervix have slowly declined as a result of regular screenings, mortality rates among many cancers remain unchanged. Lack of early detection methods and absence of clear, defined symptoms that present with early stage disease have contributed to the lack of declining mortality rates among these cancers (1). Consequently, many individuals diagnosed with cancer have dissemination of the disease at the initial time of its discovery.

For these individuals the life-threatening concern will not come from the primary site of the disease. The dissemination of cancer cells, known as metastasis, is the greatest concern, as metastasis accounts for greater than 90% of all cancer related mortalities. Once a cancer has metastasized there is no definitive cure, and in many cases clinical focus shifts from cure to slowing the spread of the disease. Stable disease, recognized as a period by which a metastatic site is not growing or spreading, is currently the most sought-after clinical achievement for patients with metastatic disease. Though many patients experience a period of stable disease, the effect is often short-lived with time to progression being defined in months. A clinical concern with these patients is that all treatment modalities will eventually be exhausted in attempting to maintain stable disease.

A great amount of effort has gone into identifying and understanding the important events and molecular players that are central to metastasis and therapy resistance. From these efforts many targets having potential for clinical benefit have been identified. However, movement of these targets into pre-clinical development has been limited as a result of many targets failing to be “druggable” by small molecules/pharmacological agents, antibodies, or ligand-binding agents. This has led to the dismissal of many worthwhile targets. One such target has been cell-cell adhesions and the individual proteins that regulate such structures. Re-expression of cell-cell adhesion proteins in carcinoma cells has the potential to improve or re-sensitize carcinoma cells to therapeutic agents. Currently, there are no known druggable schemes for re-establishing cell-cell adhesions or their individual proteins in carcinoma cells.

The work reported in this dissertation is innovative in that it establishes adenosine’s relation to the reforming of cell-cell adhesions in stressed carcinoma cells. The purinergic pathway being a highly drug targeted pathway raises the question to whether pharmacological agents could be used to re-establish cell-cell adhesions in carcinoma cells to provide therapeutic benefit in patients with metastatic disease. The purinergic pathway’s generation of adenosine and adenosine’s signaling has a history of providing epithelial cell-cell adhesion in the form of barrier function. Such epithelial barrier function is critical in maintaining homeostasis during sepsis, hypoxia, and trauma. However, the purinergic pathway is not well-studied in cancer and its exact effect on cell-cell adhesions and the individual proteins associated with such adhesions remains unclear. Using endometrial carcinoma as a model system, we have put much effort into establishing this pathway and its generation of adenosine and its relatedness to barrier function and cell-cell adhesions. The studies described within this dissertation utilize commercially available carcinoma cell lines and pharmacological agents, endometrial carcinomas obtained from FIGO stage I-IV patients, and mouse models. Overall, our objective of this work was to establish CD73’s generation of adenosine to induce barrier function in epithelial cells of the endometrium

and to establish adenosine's ability to regulate cell-cell adhesion events or proteins in carcinoma cells. This work provides the foundation and understanding of adenosine and its regulation of cell-cell adhesions in carcinoma cells, which are important to the future progression of this work into pre-clinical studies. Such movement of these findings may later lead to provide therapeutic benefit to patients with metastatic disease.

CHAPTER TWO

General Introduction

Endometrial Cancer

Carcinomas of the female reproductive tract account for almost 15% of all cancers diagnosed in women. The most common of these malignancies is uterine cancer, specifically endometrial cancer. Endometrial cancer (EC) arises in the epithelial lining of the uterus, the endometrium. In the United States an estimated 46,470 women are expected to be newly diagnosed with the disease in 2011 (1). An estimated mortality of 8,120 women is also expected. The prevalence of EC among all cancers is preceded only by cancers of the breast, lung, and colon (1). The incidence of EC over the past 10 years has continued to increase at a concerning rate (1, 2). Increased life expectancy and obesity has contributed to increased diagnoses (3, 4).

Histological subtype is a well-known independent prognostic indicator of survival in EC (5, 6). Subtypes of EC are classified according to differentiation patterns and architectural grade. The majority of ECs, 80%, are of the endometrioid subtype. Endometrioid refers to endometrial-type glands of varying differentiation that resemble proliferative-phase endometrium. Variants include secretory, villoglandular, endometrioid with squamous differentiation, and endometrioid with ciliated cells. Architectural grade divides endometrioid tumors into Grade 1, Grade 2, and Grade 3. A Grade 1 classification consists of well-differentiated glands with no more than 5% solid growth. Grade 2 endometrioid includes moderately-differentiated glands and 6-50% solid growth, and Grade 3 consists of poorly-differentiated glands and more than 50% solid growth (6). When separated by grades, Grade 3 endometrioid has the poorest prognostic outcome (6). Grade 3 survival rates are relatively similar to non-endometrioid subtypes when stage of disease is compared (6). The remaining 20% of endometrial carcinomas are represented by non-endometrioid subtypes, such as uterine papillary serous, clear cell, and malignant mixed Müllerian tumor (MMMT; carcinosarcoma). Based on the histological similarity seen among EC subtypes

and including clinical, genetic, and molecular features, EC can be divided into two broad categories, Type I and Type II disease (7-10). Type I ECs consist of low-grade, Grade 1 and Grade 2, endometrioid ECs that typically arise from a history of unopposed estrogen exposure, are of low metastatic potential (confined to the uterine corpus), and have favorable prognostic outcomes. Type II ECs includes Grade 3 endometrioid and non-endometrioid subtypes. Type II ECs appear to be un-related to high estrogen levels and are highly aggressive with metastasis common at the time of diagnosis (7). Unlike Type I that arises in a background of hyperplasia (11), Type II ECs arise in an atrophic endometrium in relatively older women. Common genetic and molecular abnormalities that occur in Type I ECs and Grade 3 endometrioid include microsatellite instability, 20-40% (12-15), inactivation of phosphatase and tensin homolog (PTEN), ~80% (16-18), and mutations in *K-ras*, 15-30% (19, 20), *PIK3CA*, 25-30% (21-24), and *CTNGB1* (encodes β -catenin), 25-40% (25-30). In contrast, Type II ECs frequently show inactivation of cyclin-dependent kinase inhibitor 2A (CDKN2A; p16), 40%, and E-cadherin, 80-90% (31), *p53* mutations, 90% (32-34), amplification of human epidermal growth factor receptor 2 (ErbB-2; Her2/neu), 10-30% (35), and chromosomal instability. While Type II abnormalities, such as *p53* mutations, E-cadherin inactivation, and *Her2/neu* amplification are seen among the endometrioid subtype, albeit in lower percentages, this is mostly attributed to Grade 3 endometrioid ECs. Not all ECs associate directly with Type I or Type II, as a limited number of ECs show mixed or overlapping features. This has led to the consideration that non-endometrioid ECs may develop by two differing pathways: (1) *de novo*, via *p53* mutation, or loss of heterozygosity at several loci; or (36) dedifferentiation from a pre-existing endometrioid ECs (9, 37). Whether either or both pathways occur is currently unclear.

Despite great advances in understanding the molecular and genetic events associated with the two types of EC and their development, very little is known regarding the molecular mechanisms of how endometrial carcinomas invade and metastasize. Consequently, the outlook

for patients with metastatic disease remains poor. The overall low mortality rate, a statistic attributed to 72% of initially diagnosed patients having uterine-confined disease cured by surgery alone (38), has overshadowed issues relating to metastatic disease, including persistence of therapy resistance and lack of targeted agents for EC. Current adjuvant therapies of combined chemotherapy, involving paclitaxel-cisplatin or doxorubicin-cisplatin, radiation, and/or hormonal therapy remain to have little to no benefit for women with metastatic EC (reviewed, (39)). Few advances have been made in identifying and developing novel therapeutic strategies for this disease. Consequently, metastatic EC patients are increasingly seeking Phase I clinical trials as a result of other options not being available for this disease. With this continuing trend, the outlook for these women will continue to be dismal.

The Effects of the Loss of Cell-Cell Adhesions in Carcinomas

The term metastasis was first proposed by Jean Claude Recamier in 1829. Since then it has been well-described that cancer cells undertake a cascade of events (metastatic cascade – initially proposed by Garth Nicolson and Jeffrey Winkelhake (40)) in order to survive and propagate in distant sites, and that the outcome of metastasis in a defined tissue or organ is not random but due to compatibility between the “seed” (cancer cells) and “soil” (metastatic site). This “seed and soil” hypothesis was first proposed by Stephan Paget in 1889 (41). Like other cancer types, ECs tend to metastasize to preferred sites, including the lung, (frequency, 41%), peritoneum and/or omentum (39%), ovary (34%), liver (29%), bowel (29%), and bladder (23%) (42). Each step of the metastatic cascade is comprised of a complex change of molecular and genetic events within the cancer cells and cells of the surrounding microenvironment. Steps of the metastatic cascade include proliferation and vascularization, invasion, embolism, transport through circulation, arrestment in organs or tissues, extravasation, establishment of a microenvironment, and proliferation and re-vascularization of the cancer cells at metastatic site(s). For a cancer cell to form a metastasis, it must successfully navigate each step (reviewed, (43)). It is believed that very few cancer cells, less than 0.01%, are able to successfully complete this cascade (44, 45).

In the initial steps of the metastatic cascade, carcinoma cells will shed their prototypical epithelial characteristics, such as cell-cell adhesions, apical-basolateral polarity, barrier function, basement membrane restriction, and cobblestone differentiation. These actions provide carcinoma cells with a phenotype that is more influencing of metastatic behavior. The loss of individual cell-cell adhesion proteins contributes to the metastatic progression of carcinoma cells. Initially recognized only for contributing to carcinoma cell migration and invasion, loss of cell-cell adhesion proteins and their structures are now known to impact proliferation and transcriptional control, basolateral receptor activity, and polarity. These events contribute significantly to the metastatic phenotype of carcinoma cells, as will be introduced with the following. Therefore, loss

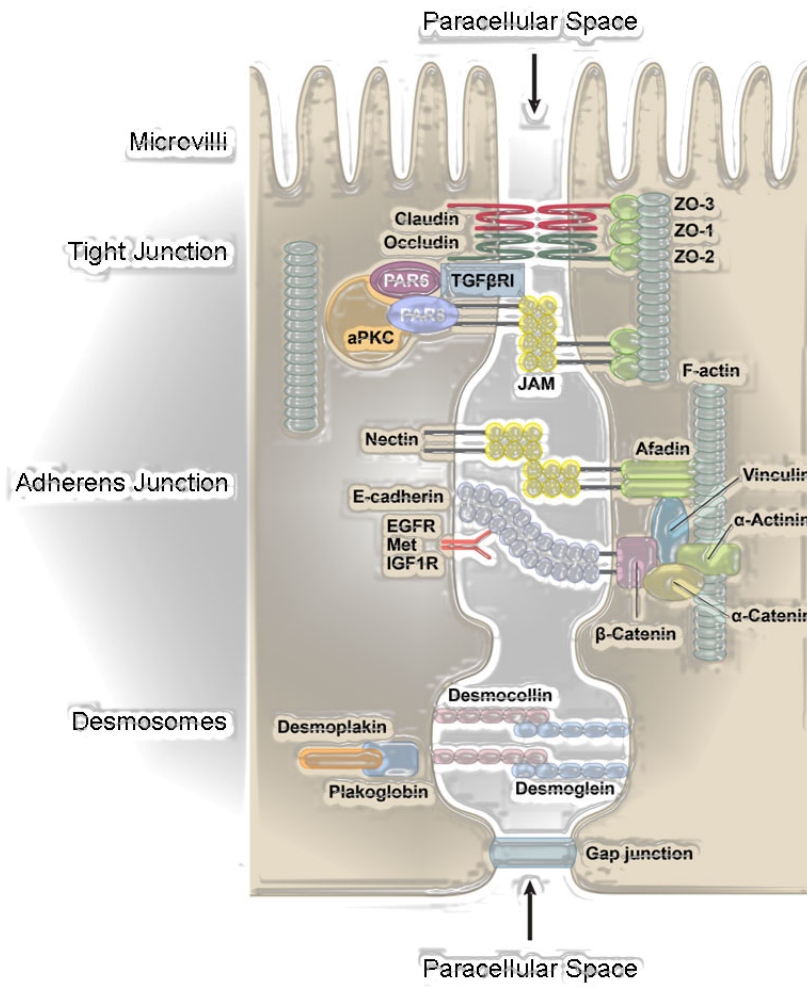
of cell-cell adhesions and their individual proteins is not a passive event in the metastatic progression of carcinoma cells. Cell-cell adhesion structures common among epithelial cells include tight junctions, adherens junctions, and desmosomes. Cell-cell adhesion structures are comprised of an array of integral membrane, peripheral plaque, and cytoskeletal associated proteins. Proteins composing cell-cell adhesion structures are illustrated in Figure 2.1 (46).

Regulation of Proliferation and Transcriptional Control by Cell-Cell Adhesions

The indication that cell-cell adhesion structures had important roles in controlling carcinoma cell proliferation and transcription of metastasis-impacting genes came from studies that identified transcription factors to be sequestered to the membrane of cell-cell adhesions. Sequestered transcription factors include ZO-1 associated nucleic acid binding protein (ZONAB), Jun, Fos, CCAAT-enhancer binding protein (C/EBP), β -catenin, and γ -catenin. ZONAB is a Y box transcription factor that regulates the G_0/G_1 to S cell cycle phase transition by interacting with cyclin dependent kinase 4 (CDK4) and inducing transcription of cyclin D1 and proliferating cell nuclear antigen (PCNA) (47-49). ZONAB also regulates the expression of the known oncogenic receptor, Her2/neu (47). ZONAB is sequestered to the membrane by peripheral plaque and tight junction protein, zona occludens 1 (ZO-1) (47). Release of ZONAB from ZO-1, as a result of ZO-1 loss or membrane delocalization, is associated with uveal melanoma cell proliferation and progression (50). Absence of ZO-1 is correlated with disease progression in breast carcinomas (51). The transcription factors Jun, Fos, activator protein 1 (AP-1), and C/EBP are bound by the peripheral plaque and tight junction protein, zona occludens 2 (ZO-2). ZO-2 contains nuclear import and export signals and therefore shuttles between the nucleus and tight junctions (52). ZO-2 can be found associated with Jun, Fos, and C/EBP in the nucleus where it acts to negatively regulate Jun, Fos, and C/EBP transcription activity (53). However, to provide function to tight junctions, ZO-2 will translocate from the nucleus to tight junctions, bringing with

Figure 2.1. Schematic summary of epithelial cell-cell adhesion structures and their associated proteins. Cell-cell adhesion structures common among epithelial cells include tight junctions, adherens junctions, and desmosomes. Cell-cell adhesion structures are comprised of a diverse array of integral membrane, peripheral plaque, and cytoskeletal associated proteins. Tight junction: integral membrane proteins, claudins, occludin, and junctional adhesion molecules (JAMs); peripheral plaque proteins, zona occludens (53); cytoskeletal associated proteins, F-actin. Adherens junction: integral membrane proteins, cadherins and nectin; peripheral plaque proteins, β -catenin, α -catenin, γ -catenin (plakoglobin), vinculin, α -actinin; cytoskeletal associated proteins, F-actin. Desmosomes: integral membrane proteins, desmocollin and desmoglein; peripheral plaque proteins, γ -catenin (plakoglobin) and desmoplakin; cytoskeletal associated proteins (not shown), intermediate filament proteins, such as keratins and vimentin. Tyrosine kinase receptors, epidermal growth factor receptor (EGFR), Met, and insulin growth factor 1 receptor (IGF1R) are bound and activity inhibited by adherens junction protein E-cadherin (54). Transport of transforming growth factor β receptor 1 to the basolateral membrane requires occludin binding (55). Occludin-TGF β 1 interact with partitioning-defective protein-6 (Par6), a polarity protein of the Par6-partitioning-defective protein 3 (Par3)-atypical protein kinase C (aPKC) polarity complex (56). Modified from Thiery, J.P. and Sleeman, J.P. Complex networks orchestrate epithelial-mesenchymal transitions, *Nature Reviews Molecular Cell Biology*, 2006, 7:131-142. Image modified courtesy of Kim Vu, Department of Pathology, the University of Texas MD Anderson Cancer Center. Image reference: (46)

Figure 2.1



it Jun, Fos, and C/EBP (53). c-Jun promotes carcinoma cell proliferation by inducing cyclin D1 and concomitantly repressing p53 transcription in the absence of ZO-2 (57, 58). Fos activation in mouse mammary epithelial cells promotes epithelial-to-mesenchymal transition (EMT), a phenotype transitioning event later described in this introduction, by induction of β -catenin/lymphoid enhancer binding factor-1 transcriptional activity (59). β -catenin is a peripheral plaque protein of adherens junctions that binds the integral membrane protein E-cadherin. β -catenin is also central to the canonical Wnt signaling pathway as a transcriptional co-regulator. Along with T-cell factor/lymphoid enhancer-binding factor 1 (TCF/LEF1) family members β -catenin can transcribe an array of proliferative and metastasis-influencing genes, such as c-myc, cyclin D1, fibronectin, matrix metalloproteinase-7 (MMP-7), cyclooxygenase 2 (COX-2), CD44, P-glycoprotein, and Snail (60) (reviewed, (61)). β -catenin stability in the cytoplasm is the determining factor for its function as a transcription factor (62, 63). Loss of E-cadherin in carcinoma cells leads to β -catenin cytoplasmic accumulation, which contributes to β -catenin's transcriptional activity (62, 63). In many cancers, including colon (64), melanoma (65), ovarian (66), hepatocellular (67), and endometrial (reviewed, (37)) single-base missense mutations of β -catenin prevent cytoplasmic degradation of β -catenin by its degradation complex, Axin-glycogen synthase kinase-3 β -adenomatous polyposis coli (Axin-GSK3 β -APC) additively contributing to β -catenin's transcriptional activities. γ -catenin, known as plakoglobin, is a peripheral plaque protein of adherens junctions and desmosomes. γ -catenin binds E-cadherin at adherens junctions and integral membrane proteins, desmocollin and desmoglein, at desmosomes. γ -catenin is a homologue of β -catenin and is found to have similar Wnt signaling roles as β -catenin (68, 69). The lack of membrane E-cadherin expression and γ -catenin nuclear accumulation correlates with lymph node metastasis and/or poor prognosis in head and neck (70), non-small cell lung cancer (NSCLC) (71), and bladder (72) cancers.

Regulation of Basolateral Receptor Activity by Cell-Cell Adhesions

At the basolateral membrane, integral cell-cell adhesion proteins, occludin and E-cadherin, directly bind to and control the activity of receptors located among cell-cell adhesion structures. These receptors include transforming growth factor- β receptor 1 (TGF β R1) (55) and receptor tyrosine kinase receptors, epidermal growth factor receptor (EGFR), Met, and insulin growth factor 1 receptor (IGF1R) (54). The loss of tight junction proteins, including claudin-1, ZO-1, and ZO-2, relieves paracellular selectivity and lends to the movement of apically sequestered ligands along the basolateral membrane. TGF β R1, known as activin receptor-like kinase 5 (ALK5), is positioned at tight junctions by occludin, an integral membrane protein of tight junctions (55). When ligand is present, active recruitment of TGF β R2 and its complex with TGF β R1 initiates signaling cascades that cause small GTPase (Rac and Rho)-induced cytoskeleton reorganization, loss of polarity, matrix metalloproteinase expression and matrix degradation, proliferation and survival, and epithelial-to-mesenchymal transition (EMT). Importantly, the activity of basolateral TGF β R1 is directly involved with disassembling tight and adherens junctions by phosphorylating occludin-TGF β R1 bound polarity protein, partitioning-defective protein-6 (Par6) which recruits an E3 ligase to target cytoskeletal assembling and cell-cell adhesion stabilizing protein, RhoA (56). EGFR, Met, and IGF1R are found among adherens junctions and bind directly with E-cadherin (54). Activity of these tyrosine kinase receptors is central to the same signaling events described for TGF β R1 activity. E-cadherin negatively regulates EGFR, cMet, and IGF1R activation in an adhesion dependent manner (54). Therefore, loss of E-cadherin from the basolateral membrane is important for the inappropriate activation of these adherens junction kinase receptors.

Regulation of Polarity by Cell-Cell Adhesions

Polarity complex proteins are required to maintain the identity of apical and basolateral orientation of cells. Collectively, cell-cell adhesion structures maintain polarity complex organization, and polarity complexes are required to maintain cell-cell adhesions. Binding of polarity complex proteins, partitioning-defective protein 3 (Par3) to tight junction protein, JAM1 and partitioning-defective protein 6 (Par6) to the occludin-TGF β RI complex is important for the assembly and apical-basolateral and basolateral positioning of polarity complexes (73). Polarity complexes and their members include, Par6, Par3, atypical protein kinase C (aPKC) (Par6-Par3-aPKC) complex; crumbs, protein associated with lin seven 1 (PALS1), PALS1-associated TJ protein (PATJ) (crumbs-PALS1-PATJ) complex; and scribble, discs large (Dlg), lethal giant larvae (Lgl) (scribble-Dlg-Lgl) complex (reviewed, (74)). The loss of JAM1, occludin, their peripheral plaque proteins, or the assembly of E-cadherin adherens junctions cause polarity complex proteins to be dispersed, non-organized or complexed in the cell. This leads to the loss of key homeostatic features including, positive feedback – location identity of proteins within the cell, membrane segregation of lipids and proteins, directed vesicle trafficking, and lipid modification. All of these processes contribute to the progression of carcinoma cells. In addition, dispersed polarity proteins are freely recruited by TGF- β , phosphoinositide-3-kinase (PI3K), and Wnt signaling to establish front-rear polarization, an event important for cell migration (reviewed, (75)). Other contributions include PALS1's inability to traffic E-cadherin to the membrane and therefore maintain cell-cell adhesions (76) and the loss of PATJ's interaction with tuberous sclerosis complex 2 (TSC2) which allows for mammalian target of rapamycin (mTOR) signaling (77).

Regulation of Migration and Invasion by Cell-Cell Adhesions

Carcinoma cells gaining the ability to migrate and invade with the absence of cell-cell adhesions or their individual proteins is a hallmark of metastasis. This migratory and invasive behavior of carcinoma cells occurs by 1.) allowing cells to simply dissociate completely or partially from one another (78, 79); 2.) relieving inhibitory control on basolateral receptor signaling (80-85); 3.) supporting re-arrangement and altering of the cell's cytoskeleton; and 4.) disrupting juxtacrine signaling that establish and maintain neighboring adhesion structures. Specific cell-cell adhesion proteins that are lost and thereby promote carcinoma cell migration and invasion include E-cadherin, claudin-1 (86), claudin-16 (87), JAM1 (88), junctional adhesion molecule 3 (JAM3) (89), and occludin (90). Decreased E-cadherin (91-94) and occludin (95) expression correlates with increased histological grade, myometrial invasion, and lymph node metastasis in EC.

Mechanisms of Cell-Cell Adhesion Loss in Carcinomas

Many mechanisms contribute to the loss of cell-cell adhesions, including epithelial-to-mesenchymal transition (EMT), mutation, epigenetic silencing, proteolysis, defective membrane transport, microRNA silencing, and tyrosine phosphorylation. EMT may be considered the most central and universal mechanism, as its activation is supported by multiple signaling pathways. Such pathways include those activated from frizzled, integrin, notch, TGF β , and/or tyrosine kinase receptors and/or serine-threonine kinase receptors. More importantly, though EMT activity is involved in the loss of cell-cell adhesions, EMT also contributes to the expression of metastasis-promoting genes (reviewed, (46)). Aside from EMT, mechanisms of cell-cell loss known to be relative in EC include mutation and epigenetic silencing. In EC, single-base missense mutations in exon 3 of the β -catenin gene, *CTNNB1*, is associated with β -catenin nuclear expression and found in 14-44% of tumors (reviewed, (37)). Mutations in the E-cadherin gene, *CDH1*, are rare in

EC (96). Epigenetic silencing has greater significance in the loss of E-cadherin in EC. DNA hypermethylation of the E-cadherin gene, *CDH1* occurs with increasing frequency in ECs that have extensive myometrial invasion, ~80% (97).

Epithelial-to-Mesenchymal Transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a genetic re-programming event that transitions a cell's lineage from an epithelial-like phenotype to a more mesenchymal-like phenotype. EMT was first recognized for its central importance in embryonic development, assisting in neural-crest formation and dorsal closure. In cancer, EMT is widely accepted as being a hijacked event of carcinoma cells that promotes metastasis by creating a more migratory and invasive behavior in cells. This is attributed to the mesenchymal-like phenotype and matrix degradation activities. EMT hallmarks include cell-cell adhesion down-regulation by transcriptional suppression of cell-cell adhesion genes, transcriptional up-regulation of matrix degrading proteases and mesenchymal-related proteins, such as N-cadherin and vimentin, and Rho-mediated actin cytoskeleton rearrangement (reviewed, (46)). The genetic re-programming of EMT is mediated by a collection of EMT transcription factors, including snail (SNAI1) (98, 99), slug (SNAI2) (100, 101), δ EF1 (ZEB1) (102), SIP1 (ZEB2) (103), E12/E47 (104), and twist (105). Cell-cell adhesion proteins directly targeted by EMT transcription factors include E-cadherin, occludin, claudin-1 and claudin-7 (106, 107). Transcriptional silencing occurs by EMT transcription factors binding to E-box consensus sequences, CANNTG, in the promoter of target cell-cell adhesion proteins. In EC, SNAI1 (108) and ZEB1 (109) are found to correlate with Type II ECs and membrane-bound loss of E-cadherin expression. In most carcinoma types, EMT is activated by combined stimulation of frizzled, integrin, notch, TGF β , tyrosine and/or serine-threonine kinase receptors. Signaling pathways that activate EMT include Ras-Raf-MEK-MAPK, PI3K-Akt, TGF β -Smads, ILK-Akt, Wnt- β -catenin, TGF β -Par6-Smurf (reviewed, (46)),

abbreviations: MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; integrin-linked kinase (ILK).

Adding to the Collective Effects of the Loss of Cell-Cell Adhesions in Carcinomas –

Therapy Resistance

In the last several years, the effects from the loss of cell-cell adhesions, such as proliferation and transcriptional control, basolateral receptor activity, polarity, and migration and invasion, have been expanded to include therapy resistance. In carcinoma cells, including non-small cell lung cancer (NSCLC) (110, 111), breast (112), colon (113, 114), pancreatic (114), urothelial (115), epidermoid (116), and liver (116), sensitivity to chemotherapy agents (cisplatin and oxaliplatin) and epidermal growth factor (EGFR) inhibitors (gefitinib, erlotinib, and cetuximab) significantly correlates with the presence of cell-cell adhesion proteins, E-cadherin (110-115), β -catenin (113, 115), α -catenin (110), γ -catenin (110, 113, 114, 116), and claudin-7 (110). α -catenin is a peripheral plaque protein of adherens junctions. Cisplatin is one of the most widely used chemotherapy agents and shows efficacy in cancers, such as head and neck, NSCLC, cervical, bladder, ovarian, and endometrial (reviewed, (117)). Cisplatin acts by cross-linking DNA (118). EGFR inhibitors (cetuximab, a monoclonal antibody; gefitinib and erlotinib, tyrosine kinase inhibitors), have shown clinical effectiveness for NSCLC (119, 120), colon (121), pancreatic (122), and head and neck (123) cancers. EGFR is frequently overactive in many cancer types, leading to abnormal activation of pro-proliferative and anti-apoptotic responses through phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and Ras/Raf/Mek signaling (124). The dependence of cell-cell adhesion proteins on therapy responsiveness has been established in NSCLC (111), urothelial (115), and breast (112) carcinoma cells. Resistant clones to cisplatin and/or EGFR inhibitors are re-sensitized to therapy by transfection of E-cadherin. The reverse occurs with siRNA targeting of E-cadherin, as carcinoma cells once sensitive become resistant. In cisplatin

resistant clones of epidermoid and liver carcinomas sensitivity to cisplatin is restored by transfection of γ -catenin (116). Importantly, clinical relevance of cell-cell adhesion protein presence and therapy sensitivity has been established. This has been demonstrated in NSCLC patients receiving the EGFR inhibitor, erlotinib. TRIBUTE, a Phase III randomized, placebo-controlled trial conducted in the United States, enrolled chemotherapy-naïve patients with metastatic NSCLC to compare disease progression and survival rates of patients who received erlotinib combined with cisplatin and paclitaxel to patients who received cisplatin and paclitaxel. NSCLC patients with E-cadherin positive tumors have significantly longer time to progression (hazard ratio 0.37; log rank $p=0.0028$) compared to patients with E-cadherin negative tumors (110). A trend of longer survival was also found for E-cadherin positive patients (110). Known mechanisms for cisplatin resistance include changes in cellular uptake and efflux of the agent, increased detoxification, inhibiting of apoptosis signals, and increase in DNA repair (reviewed, (118, 125)). Mechanisms relating to how and why loss of cell-cell adhesion proteins lead to therapy resistance are less known. One assumption, involving cisplatin resistance with γ -catenin loss, is that dissociation between the actin cytoskeleton and membrane cause altered location of membrane proteins important for drug uptake (116). Observations of declined rates of endocytosis with loss of cell-cell adhesion proteins are also expected to contribute to resistance (116).

My interest in the regulation of cell-cell adhesions involves the therapeutic potential of re-establishing such contacts to improve drug sensitivity. In EC, the mainstay therapies for metastatic disease are combination chemotherapy regimens that involve cisplatin, such as paclitaxel-cisplatin or doxorubicin-cisplatin (reviewed, (39)). With all metastatic cancers, resistance to therapy or acquired resistance is a significant clinical issue. A significant limitation to targeting cell-cell adhesions and their individual proteins, such as E-cadherin, in a clinical setting has been the inability to target these structures and proteins by small

molecules/pharmacological agents, antibodies, or ligand-binding agents. Currently, there are no known druggable signaling pathways that work to re-establish cell-cell adhesions or their individual proteins in carcinoma cells. This important clinical observation has led to our consideration of the purinergic pathway, a pathway that is drug targeted in a variety of different diseases. The purinergic pathway's generation of adenosine and its signaling activity has a history of providing epithelial cell-cell adhesion in the form of barrier function. Barrier function is a homeostatic event of endothelial and epithelial cells and its actions are directly related to the regulation and maintained integrity of cell-cell adhesions and their individual proteins. The remainder of this introduction will introduce the purinergic pathway and adenosine and adenosine's tissue protective effect of barrier function.

Purinergic Pathway

A great amount of research laboratory-based effort has been devoted to identifying and understanding the important molecular events that are central to metastasis and therapy resistance, as these mechanisms are the primary causes of cancer morbidity and mortality. From these efforts many potential therapeutic targets have been identified. Unfortunately, many of these potential targets have not progressed beyond pre-clinical testing because currently available pharmacological agents cannot be directed against them. Examples include the cell-cell adhesion proteins, E-cadherin and occluding; re-expression of these proteins in cells *in vitro* reverses therapeutic resistance and/or phenotypic changes associated with EMT. Additional examples include EMT transcription factors, which are central to the genetic re-programming of carcinoma cells. Various pharmacological agents already exist for members of the purinergic pathway (reviewed, (126)), so this metabolic pathway is targeted in a variety of diseases, such as asthma, chronic obstructive pulmonary disease, interstitial lung disease, Huntington's disease, Parkinson's disease, and congestive heart failure (reviewed, (126)). The significance of the purinergic pathway in cancer has not been studied in great detail, and therefore the potential for available drugs that target this pathway to provide therapeutic benefit in cancer patients has not been adequately explored. Our interest in the purinergic pathway and its generation of adenosine in EC involves its ability to induce barrier function. Barrier function is directly associated to the regulation and maintained integrity of cell-cell adhesions and their individual proteins.

The purinergic pathway is a ubiquitously expressed metabolic pathway comprised of various cell surface and intracellular localized enzymes, membrane-oriented nucleoside transporters, and G-protein coupled and ligand-gated cation channel receptors that is primarily recognized for its generation and regulation of purine nucleotides and nucleosides. Adenine nucleotides, such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), and the nucleoside adenosine are the best-described components of this

pathway. Along with being central to energy metabolism, adenine nucleotides or nucleosides can exert substantial effects on various tissues and cell types at the extracellular level. Allen Drury and Albert Szent-Györgyi were the first to show such a response, in 1929, as intravenous injection of tissue isolated purines, AMP and adenosine, caused arterial dilation, lowering of blood pressure, and temporary heart block (127). Purine receptors were not recognized until decades later following an accumulation of studies concerning the differential effects of extracellular purines. It was Geoffrey Burnstock that defined the basis for distinguishing the two main types of purine receptors, P1, selective for adenosine and P2, selective for ATP and ADP (128). Separation was based on relative potencies of ATP, ADP, AMP, and adenosine, selective antagonistic effects of adenosine by methylxanthines (129), activation of adenylate cyclase by adenosine, and stimulation of prostaglandin synthesis by ATP and ADP (128). P2 receptors have since been further classified into two families based on inherent biological properties of the receptors and are defined as P2X, ligand-gated cation channel receptors (130), and P2Y, G protein-coupled receptors (131, 132). No studies at this time indicate P1 or P2 receptors to be responsive to AMP. Likewise, an unknown receptor for AMP has not been identified.

Adenine nucleotides, ATP and ADP, gain access to the extracellular surface of cells by disrupted membrane integrity, exocytosis, and channel-mediated efflux (133). The release of ATP and/or ADP is enhanced by cellular stressors, such as hypoxia, ischemia, and cellular stretch and swelling. Channel-mediated efflux includes the cystic fibrosis transmembrane conductance regulator (CFTR) (134, 135), multidrug resistance-1 p-glycoprotein (MDR1-Pgp) (136), volume-sensitive outwardly rectifying (VSOR) anion channels (137), maxi-anion channels (138), voltage-dependent anion channel-1 (VDAC-1) (139), and connexin hemichannels (140-142). The purine nucleoside adenosine can gain access to the extracellular membrane in a similar manner by accessing equilibrative nucleoside transporters (ENTs) or sodium-dependent concentrative nucleoside transporters (CNTs). In basal physiology conditions, extracellular adenosine is largely

the result of adenosine's passive transfer through equilibrative nucleoside transporters (143). In contrast, with cellular stress or disease the main source of extracellular adenosine comes from phosphohydrolysis of liberated adenine nucleotides (144, 145). Ecto-enzymes responsible for phosphohydrolysis of ATP, ADP, and AMP include the following:

- 1.) nucleoside triphosphate diphosphohydrolase (NTPDases), which catalyze adenine nucleotide phosphohydrolysis $ATP \rightarrow ADP \rightarrow AMP$;
- 2.) nucleotide pyrophosphatase/phosphodiesterases (NPPs), which catalyze adenine nucleotide phosphohydrolysis $ATP \rightarrow ADP \rightarrow AMP$;
- 3.) alkaline phosphatases, which catalyze adenine nucleotide phosphohydrolysis $ATP \rightarrow ADP \rightarrow AMP \rightarrow$ adenosine;
- 4.) 5'nucleotidase (CD73), which catalyzes adenine nucleotide phosphohydrolysis $AMP \rightarrow$ adenosine.

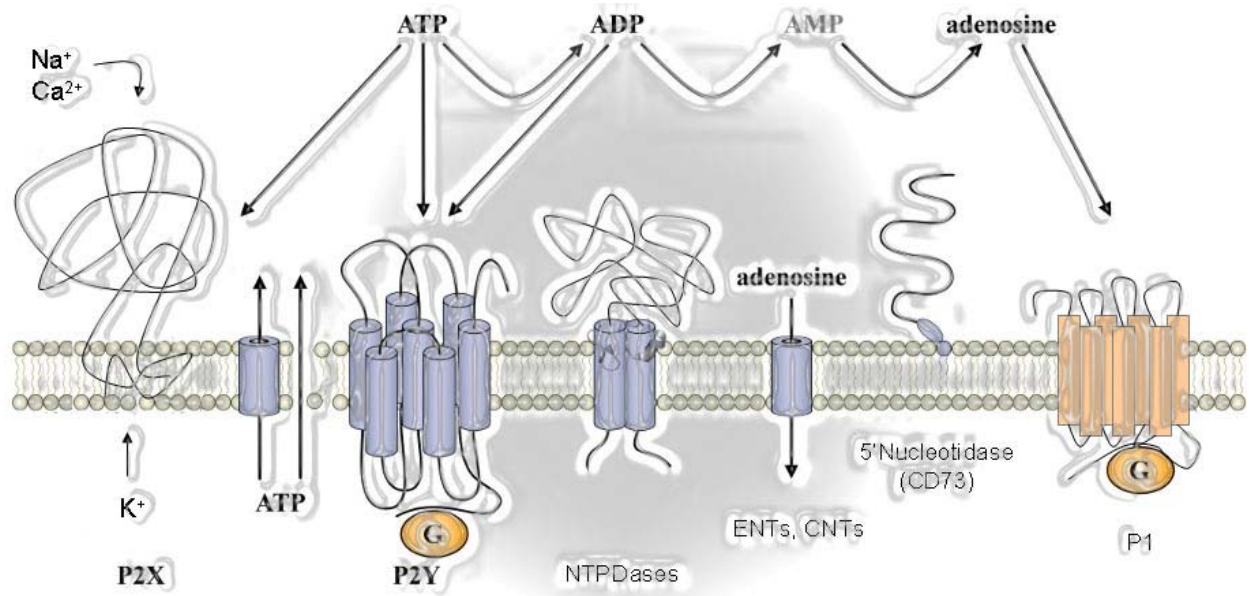
Activity of the various ecto-enzymes is dependent on cell type, availability and concentration of co-activators (Ca^{2+} and Mg^{2+}), individual Michaelis–Menten kinetics (K_M), and local concentrations of ATP, ADP, and AMP (reviewed, (146)). A schematic summary of extracellular members of the purinergic pathway and phosphohydrolysis of adenine nucleotides at the extracellular surface is detailed in Figure 2.2.

Purinergic Pathway Ecto-enzyme 5'Nucleotidase (CD73)

5'Nucleotidase (CD73) is a Zn^{2+} dependent ecto-enzyme that catalyses the phosphohydrolysis of purine and pyrimidine ribo- and deoxyribonucleoside 5'monophosphates to their respective nucleosides. 5'AMP is CD73's preferred substrate with K_M values of 3-6 μ M (147-149). CD73 is found essentially in all tissue types. However, the distribution of the enzyme

Figure 2.2 Schematic summary of extracellular purinergic pathway members and adenine nucleotide phosphohydrolysis. Adenine nucleotides, ATP and ADP, gain access to the extracellular surface by disrupted membrane integrity, exocytosis, or channel-mediated efflux. ATP acting on P2X ligand-gated cation channel receptors or P2Y G protein-coupled receptors can initiate a diverse array of intracellular signaling networks. P2X and P2Y receptors are nucleotide specific. Membrane associated ecto-enzymes, such as NTPDases and 5' nucleotidase (CD73), convert ATP or ADP to AMP and AMP to adenosine by phosphohydrolysis, respectively. Generated adenosine can initiate equally diverse intracellular signaling networks by acting on P1 G protein-coupled receptors. Adenosine is the primary agonist of P1 receptor subtypes. Modified and from the DFG Research Group, Neuronal and glial P2 receptors molecular basis and functional significance, www.uni-leipzig.de/~biochem/for748/index.php. Image modified courtesy of Kim Vu, Department of Pathology, the University of Texas MD Anderson Cancer Center. Image reference: (150).

Figure 2.2



is not always homogeneous in a particular tissue, even among cellular residents (151-153). CD73 resides in glycolipid-rich membrane subdomains known as lipid rafts and is anchored by a C-terminal glycosyl phosphatidylinositol (GPI) linkage (154). The physiological relevance of CD73's membrane anchorage by GPI is not understood. CD73 is synthesized in the rough endoplasmic reticulum as an initial precursor. Co-translational modification, such as N-linked glycosylation (155, 156), and proteolytic cleavage of the C-terminal hydrophobic domain and simultaneous GPI replacement forms the mature protein (154). The amount of CD73 at the cell surface and within intracellular pools varies among cell types and is constantly recycled from the cell surface to intracellular pools and back. The well-described function for CD73 is catalytic generation of adenosine (reviewed, (157)). Functions of CD73 that are not related to its catalytic activity have been described and include co-stimulatory molecule for the CD3/T-cell receptor (TCR) (158-162) and cell adhesion. Cell adhesion studies are less developed and have included CD73's interaction with extracellular matrix (ECM) proteins laminin, fibronectin (163, 164), and tenascin C (165). CD73 expression can be regulated by retinoic acid, interferon- γ (INF- γ) (166), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) (167).

The Fate of Extracellular Adenosine

Once generated by 5'nucleotidase (CD73), adenosine has the following four possible fates: 1.) internalization through nucleoside transporters, ENTs and CNTs; 2.) deamination to inosine by the extracellular CD26-bound adenosine deaminase (ADA); 3.) phosphorylation to AMP by adenosine kinase (AK); 4.) activation of P1 receptor subtypes. P1 receptor subtypes include adenosine 1 receptor (A1R), adenosine 2A receptor (A2AR), adenosine 2B receptor (A2BR), and adenosine 3 receptor (A3R). The fate of adenosine depends upon a combination of situations including local adenosine concentration, presence and density of P1 receptor subtypes, individual binding affinities of specific P1 receptors, presence of nucleoside transporters, ENTs

and CNTs, and presence of ecto-enzymes, CD26-ADA and AK. The fate of adenosine is also affected by the overall cellular state of a cell. Cellular stressors, such as inflammation, ischemia, hypoxia, or disease can influence the fate of adenosine. Increased levels of extracellular adenosine, such as the 100 μ M levels seen in asthmatics (168), typically occurs with disease or cellular stress. Estimated extracellular adenosine concentrations at basal physiology is 40-600nM (169).

P1 Receptor Subtypes, Adenosine Receptors

The action of extracellular adenosine to activate a receptor was first considered based on the observation by Taisija De Gubareff and William Sleator, Jr., that adenosine's action in the heart could be antagonized by methylxanthines, including caffeine and theophylline (170). Four G-protein coupled receptors for adenosine have since been described, A1R (171), A2AR (172), A2BR (173), and A3R (174), and are individually or collectively expressed in all tissue and cell types (reviewed, (175)). In this introduction thus far the adenosine receptors have been collectively referred to by their broad P1 classification. Among scientists studying adenosine, however, these receptor types are more classically viewed as belonging to one of two categories, those that stimulate adenylate cyclase and those that inhibit its action. Subtypes A2AR and A2BR are coupled to adenylate cyclase and stimulate the increase of cyclic adenosine monophosphate (cAMP), while A1R and A3R subtypes inhibit adenylate cyclase and raise intracellular Ca²⁺ concentrations. The G proteins G_i, G_o, G_q, and G_s are known to selectively couple to the adenosine receptor subtypes, thereby controlling the specificity of the signaling network that is activated by a particular subtype (reviewed, (175)). Further specificity of the activation of a particular signaling network is achieved by differences in binding affinity for adenosine among subtypes, tissue or cell type specific expression of a subtype, and tissue or cell type specific expression of G proteins. Adenosine's affinity for adenosine receptor subtypes is

A1R>A3R>A2AR>A2BR. Table 2.1 details individual affinities for the adenosine receptor subtypes.

Adenosine Receptor Subtype, A2BR

The structure of A2BR is typical of G protein-coupled receptors, with seven transmembrane domains connected by three extracellular and three intracellular loops, and flanked by an extracellular N-terminus and an intracellular C-terminus. A2BR is considered the low affinity adenosine receptor and is therefore understood to be activated only at exceptionally high concentrations of adenosine as those typical of pathological conditions rather than physiological, ~10 μ M or higher (176). Gene transcript and radioligand studies have identified A2BR to be expressed in various tissues, including the uterus (177, 178). A2BR is coupled to G_s and G_q families of G proteins. Coupled-G_s is associated with adenylyl cyclase activity, cAMP accumulation, and protein kinase A (PKA) activity. Coupled-G_q is associated with phosphatidylinositol-phospholipase C (PI-PLC) and increased diacylglycerol (DAG) and protein kinase C (PKC) accumulation and inositol triphosphate (IP3) and Ca²⁺ mobilization (reviewed, (179)). A2BR signaling also converges with the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3'-kinase (PI3K) pathways in a cAMP-dependent and PKA-dependent or – independent manner (180).

The Tissue Protective Nature of Adenosine – Barrier Function

The tissue protective nature of adenosine was first recognized by adenosine's ability to cause coronary artery vasodilation in response to hypoxia. Vasodilation allows for more oxygenated blood to be delivered to hypoxic tissues. Adenosine has since evolved as an important signaling molecule that provides homeostatic function and tissue protection in normal tissues in response to cellular stressors, such as inflammation, ischemia, and hypoxia. With all cellular

Table 2.1. Individual binding affinities for the adenosine receptor subtypes

Adenosine Receptor	Adenosine Affinity
A1R	^a 15-100nM
A2AR	^a 0.5-20 μ M
A2BR	^b >10 μ M
A3R	^c 30-300nM

^aReference: (181)

^bReference: (182)

^cReference: (183)

stressors the increased production of adenosine at the extracellular surface and/or adenosine's release into the microenvironment is the central theme to adenosine's tissue protective effects. The outcome of adenosine's effects therefore depends on the target tissue or cell type and adenosine receptor subtypes activated. Tissues protective effects include stimulating immunosuppressive responses, activation of vasodilation, ischemic pre-conditioning and post-conditioning, balancing the oxygen supply/demand ratio, inducing endothelial and epithelial barrier function, inhibiting superoxide generation, and inhibiting excitatory neurotransmitter release (reviewed, (184)). Though all have great importance in re-gaining and maintaining the basal physiology of tissues, adenosine's tissue protective effect of barrier function will be the central focus of this dissertation.

Barrier Function

Barrier function is a homeostatic event of endothelial and epithelial cells whose actions are directly related to the regulation and maintained integrity of cell-cell adhesions and their individual proteins. Barrier function is a feature specific to cell types that form a biological barrier between the outside (lumen) and inside (interstitial tissue) environments of a tissue. Endothelial and epithelial cell types have important barrier functions. The objective of this barrier is to regulate the movement of ions, solutes, and H₂O and prevent the movement of noxious stimuli, such as pathogens, bacteria, and foreign antigens through the spaces between the cells (paracellular space). Cell-cell adhesions are the primary basis of barrier function, as 1.) their integral membrane proteins serve as the physical barrier of the paracellular space and 2.) select integral proteins actively regulate the permeability state of the cells. Of the latter, the tight junction proteins occludin and claudins are the main proteins that define selected permeability (reviewed, (185)). In response to physiological needs or stressors, barrier function activation is an effort of extracellular factors to communicate with intracellular mediators to make structural

changes to the actin cytoskeleton and/or cell-cell adhesions in order to respond appropriately. The response may be a simple change in permeability or an abrupt recovery effort of cell-cell adhesions following stressor-induced disruption. Extracellular factors that accumulate or are released with cellular stress and initiate barrier protection include cytokines, such as interleukin-10 (IL-10) (186, 187) and TGF- β (188-193), growth factors, such as epidermal growth factor (EGF) (194) and hepatocyte growth factor (HGF) (195), and small molecules, such as adenine nucleotides and adenosine (196).

Barrier Function – CD73-generated Adenosine and Adenosine's Action on A2BR

Indirect observations provided the first indications that adenosine provided barrier function. For example, adenosine protected against lung microvascular leakage produced by phorbol myristate acetate (PMA) (197), adenosine blocked ischemia-reperfusion induced permeability and damage of endothelial cells (198), and endothelial cells exposed to adenine nucleotides and adenosine caused intracellular cAMP accumulation (199), a second messenger known to regulate endothelial permeability (200, 201). The direct association between adenosine and barrier function was shown by Frederick Haselton in 1993 (202), who demonstrated that applied adenosine to bovine fetal aortic endothelial cells (BAEC) decreased permeability to paracellular tracers in a reversible, concentration-dependent manner; through the use of receptor specific adenosine analogs, this effect was demonstrated to be mediated via A2 receptors subtypes A2AR or A2BR (202). Adenosine's relation to cellular stress was discovered in separate studies by Lois Richard (203) and Paul Lennon (196). Since then, adenosine's activation of barrier function has been described in the context of various stressors, cell types, tissue systems, and diseases. The present understanding of adenosine's induction of barrier function will be presented by emphasizing the studies that have advanced this understanding.

Stressor → AMP → CD73 → Adenosine → A2BR → Barrier Function

Louis Richard's work had demonstrated that application of adenosine prevented the disruption of barrier function in oxidant-injured human umbilical vein endothelial cell (HUVEC) monolayers (203). However, Paul Lennon was the first to show that adenine nucleotides and adenosine released from an *in vitro* stressor, activated polymorphonuclear leukocytes (PMNs), resulted in barrier function. Prior to these studies, adenosine's effect on barrier function had only been demonstrated by physical addition of adenosine to cell monolayers (196). Co-culture studies showed endothelial expressed CD73 to be responsible for converting PMN-released AMP to adenosine and adenosine to activate A2BR to induce barrier function (196). Therefore, these studies were important in defining that 1.) adenosine accumulated at the extracellular surface as a result of its release and CD73's conversion of AMP to adenosine, 2.) a stressor, activated PMNs, physically lead to adenosine's induction of barrier function, and 3.) CD73-generated adenosine activated A2BR to induce barrier function.

The first studies showing this same effect, **Stressor → AMP → CD73 → Adenosine → A2BR → Barrier Function**, with hypoxia came from separate studies from Kristin Synnestyedt (204), and Holger Eltzshing (144). By co-culturing activated PMNs with normoxic or post-hypoxic conditioned human microvascular endothelial cells (HMEC-1), post-hypoxic HMEC-1 were had a greater than a 60% increase in barrier function than normoxia conditioned HMEC-1 (144). These studies further demonstrated adenosine's activation of A2BR to be important for the increased barrier function (144). Kristin Synnestyedt's work was the first to show relevance of hypoxia to induce adenosine's barrier function in intestinal epithelial cells (204). Mice gavaged with α,β -methylene diphosphate (AOPCP), a catalytic inhibitor of CD73, had increased epithelial intestinal permeability, as shown by FITC-tracer leakage from the intestinal lumen into the blood, when placed in hypoxia (204). Similar studies with endothelial cells in CD73 deficient (CD73^{-/-}) (205) and A2BR deficient (A2BR^{-/-}) (206) mice have since been completed. The concept that

adenosine's promotion of barrier function is most important under conditions of stress is best highlighted by the work in the CD73^{-/-} and A2BR^{-/-} mice. These mice are both phenotypically normal under basal conditions. However, both mice suffer extensive vascular leak and pulmonary edema when placed in hypoxic conditions (205, 206).

Adenosine's Barrier Function Effect is Supported by Increased Expression of CD73 and A2BR and Down-Regulation of ENT1

From the above studies, two central themes are evident. One, adenosine's barrier function is a product of accumulated extracellular adenosine. Two, adenosine's barrier function is a product of CD73's generation of adenosine and its activation of the adenosine receptor subtype, A2BR (**Stressor → AMP → CD73 → Adenosine → A2BR → Barrier Function**). A third central theme is that adenosine's barrier function effect is supported by shifts in expression of purinergic pathway member expression. This third theme is derived from studies demonstrating that with stress, cytokine release from inflammatory cells or increased steady-state levels of hypoxia-inducible factor-1 α (HIF-1 α) controlled the expression of molecules known to be critical in adenosine generation and regulation. One of the earliest findings had shown the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) (207) and A2BR paracrine signaling (AMP → CD73 → Adenosine → A2BR signaling → CD73 transcript expression) (208) to increase CD73 expression to prepare for resealing disrupted endothelial barriers by extravasated PMNs (207) or to protect against the disruption of barrier function (208). Though unrelated to barrier function or endothelial or epithelial cells, studies by Shuichi Kobayashi (209) were among the first to show the global shifts in purinergic pathway members in response to hypoxia. This shift was supportive of the extracellular accumulation of adenosine. Shown in rat adrenal pheochromocytoma cells (PC12), hypoxia caused significant decreases in adenosine deaminase (ADA), adenosine kinase (AK), and equilibrative nucleoside transporter (ENT1) expression, while CD73 expression

significantly increased. It was concluded that accumulation of extracellular adenosine was associated with hypoxia, but the authors were unclear of its importance (209). Kristin Synnestvedt's studies in intestinal epithelial cells were the first to show that CD73's increased expression in hypoxia was a result of HIF-1 transcriptional induction of CD73 (204). Holger Eltzshing's post-hypoxia human microvascular endothelial cells (HMEC-1) studies also indicated that CD73 and A2BR mRNA was increased by 12- and 5-fold, respectively, over normoxia conditioned HMEC-1 (144). It has since been found that many members of the purinergic pathway are up-or down-regulated by HIF-1 in hypoxia to support the accumulation of extracellular adenosine for barrier function. These include CD73 (up-regulated) (204), A2BR (up-regulated) (210), ENT1 (down-regulated) (211), AK (down-regulation) (212).

Intracellular Mediators of Adenosine's Barrier Function – The Gap in the Knowledge

Adenosine's generation by CD73 and adenosine's activation of A2BR have been well-established to induce barrier function. However, it remains largely unknown which intracellular mediators are activated by adenosine's activation of A2BR. cAMP, a central secondary messenger of many G protein-coupled receptors including A2BR, is associated with the activation of intracellular barrier function mediators, PKA and exchange protein directly activated by cAMP (Epac). PKA activation inhibits GTPase, RhoA activation of cell contractility (213). Cell contraction, a barrier disruption action, is related to actin/myosin-driven force that pulls adherens junctions inward, forcing them to dissociate from their adjacent partner (214). Epac1 and Epac2 are cAMP-dependent guanine-nucleotide-exchange factors for the small GTPases Rap1 and Rap2, and are known to be important mediators of cAMP signaling (215, 216). Epac increases the integrity of adherens and tight junctions by enhancing the distribution of member proteins to cell-cell adhesion sites (217). Additional mechanisms that are related to the known down-stream signaling events of A2BR have included activation of PKC (218) and alterations in intracellular

Ca²⁺ (219). Though many of these mechanisms are intuitively related to A2BR signaling, these have yet to be convincingly related to adenosine's barrier function. Studies from Donald Lawrence and Katrina Comerford have provided the most insight into how A2BR activation may be regulating endothelial (220) and epithelial (221) barrier function. Such studies have described a PKA-dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP). VASP is a protein involved with actin cytoskeleton remodeling (222). Upon phosphorylation, VASP localizes to tight junction structures and associates with ZO-1. This leads to the structural relaxation of the actin cytoskeleton which is needed to re-form disrupted cell-cell adhesion structures (220, 221). VASP is a major target for cyclic nucleotide kinases, including PKA.

Concluding Remarks

The dissemination of cancer cells and their propagation in an organ or tissue is the most life-threatening issue of cancer as there is no definitive cure for metastatic disease. Likewise, therapy resistance is a significant clinical issue for patients with metastatic disease. Currently, there are no mechanisms that can re-sensitize cancer cells to a therapy. The work reported in this dissertation is innovative in that it establishes adenosine's activation of A2BR to be related to the reforming of cell-cell adhesions in stressed carcinoma cells. The purinergic pathway is a highly drug targeted pathway, which raises the possibility that pharmacological agonists of A2BR could be used to re-establish cell-cell adhesions in carcinoma cells and therefore provide therapeutic benefit (re-sensitize to therapy) in patients with metastatic disease. Targeting cell-cell adhesions *in vivo* has been an area of great interest, but clinically useful strategies have been lacking. The concept of adenosine-mediated cell-cell adhesion has not been previously reported for carcinomas of any tissue type. Therefore, the focus of this dissertation was to establish this association in EC. Defining the basic science behind these cellular events in cancer is valuable, as it will provide the rationale for proceeding with pre-clinical studies. Such studies are labor-, time-, and resource-

intensive. With an increased pressure to provide new therapeutic strategies for clinical use, novel ideas that are applicable and relevant need to be established in the research laboratory.

From the basic science perspective, this dissertation work is exciting as it describes the very novel discovery that adenosine's activation of A2BR induces filopodia that have cell-cell adhesion function. The function of filopodia promoting cell-cell adhesions is well-described in embryonic development and with wound healing (223-225). Such an event is important for the proper alignment and development of mature adhesion structures. The gap in knowledge in the adenosine field concerning adenosine's barrier function activity has been the mechanism by which it regulates cell-cell adhesions. Few studies have shed light on this topic (220, 221). Our work here, describing adenosine's regulation of cell-cell adhesion functioning filopodia, will hopefully help to provide a better understanding of adenosine's role in cell-cell adhesions both in cancer and in other non-cancer systems, such as trauma and sepsis. This work significantly adds not only to the understanding of adenosine's barrier function in the adenosine field, but it introduces a novel mechanism (adenosine-mediated re-forming cell-cell adhesions) that has to be avoided/down-regulated by carcinoma cells attempting to lose attachment with their neighboring cells.

CHAPTER THREE

Materials and Methods

Normal Tissues and Carcinomas

Endometrial and ovarian normal tissues and carcinomas were received from The University of Texas MD Anderson Cancer Center Multidisciplinary Gynecological Cancer Translational Research Tumor Bank (MDA-GCTR-TB). Hematoxylin and eosin stained slides were microscopically reviewed by a gynecological pathologist, Dr. Russell Broaddus, to confirm surgical stage, tumor grade, and histotype in accordance to the criteria established by the International Federation of Gynecology and Obstetrics (FIGO). Colon carcinomas with matched adjacent normal colon tissue were received from The University of Texas MD Anderson Cancer Center Tissue Biospecimen and Pathology Resource (TBPR) Institutional Tissue Bank. The TBPR is a Cancer Center Support Grant (CCGS) supported core facility funded by NCI #CA16672. Use of human tissues was approved by The University of Texas MD Anderson Cancer Center Institution Review Board, LAB01-718.

RNA – Human Endometrial Biopsies

RNA was obtained from human endometrial biopsies of premenopausal women who received depot medroxyprogesterone acetate (MPA) 150mg intramuscular injection. These biopsies were part of a clinical trial assessing the effectiveness of hormonal therapy. Baseline (pre-treatment) and 3 month post-treatment endometrial biopsies were obtained. For baseline and post-treatment, endometrial biopsies were timed to occur on days 8 or 9 of the menstrual cycle. These biopsies were timed as such to occur during the proliferative phase of the endometrium. RNA from these biopsies were kindly provided by Dr. David Loose, Department of Integrative Biology and Pharmacology, the University of Texas at Houston - Medical School, Houston, Texas.

RNA – Platinum Sensitivity

RNA was obtained from platinum sensitive and platinum resistant ovarian high grade papillary serous carcinomas (HGpsc) (226). Resistance or sensitivity was defined by review of medical records and resistance criteria set forth by the Gynecological Oncology Group, which included the following: 1.) disease progression while on a first-line platinum-based regimen; 2.) disease progression within 6 months of completion of platinum-based therapy; and 3.) persistent clinically measurable disease with best response as stable disease at the completion of first-line therapy

Animals

C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). 5' nucleotidase (CD73) (205) and adenosine receptor 2B deficient mice (227), indicated as CD73^{-/-} and A2BR^{-/-}, were provided by Dr. Michael Blackburn, Department of Biochemistry and Molecular Biology, the University of Texas at Houston - Medical School, Houston, Texas. Animals were maintained in accordance to the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Care (AAALAC)

Carcinoma Cell Lines

Endometrial

Endometrial carcinoma cell lines HEC-1A, HEC-1B, KLE (228), ECC-1, and AN3CA were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Ishikawa cells were kindly provided by Changping Zhou, Ph.D., The University of Connecticut Health Center, Farmington, CT. HEC-1A cells were cultured in McCoy's 5A medium (Cellgro, Manassas, VA) containing 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin. HEC-1B, Ishikawa, and AN3CA cells were cultured in Minimum Essential Medium (MEM) (Cellgro,

Manassas, VA) with 10% FBS, 1X Na⁺ pyruvate, 1X non-essential amino acids, and 1X penicillin-streptomycin. ECC-1 and KLE were cultured in DMEM (Cellgro, Manassas, VA) with 10% FBS, 1X Na⁺ pyruvate, and 1X penicillin-streptomycin.

WISH-HeLa

WISH-HeLa cells were kindly provided by Dr. Daniel Carson, Rice University, Houston, Texas. WISH-HeLa cells were cultured in DMEM (Cellgro, Manassas, VA) with 10% FBS, 1X Na⁺ pyruvate, and 1X penicillin-streptomycin.

RNA Isolation

Normal and Carcinoma Tissues

Total RNA was isolated from colon, endometrial, and ovarian normal tissues and carcinomas using a phenol-based method. Tissues and carcinomas were homogenized via a polytron in TRIzol® Reagent (Invitrogen, Carlsbad, CA) and mixed with chloroform. Lysates were separated into three phases by centrifugation. Total RNA was precipitated from the aqueous phase with isopropanol, washed with 75% DEPC-ethanol, and re-suspended in DEPC H₂O. Isolated RNA was DNase (Roche, Mannheim, Germany) treated per manufacturer instructions.

Carcinoma Cell Lines

Total RNA from carcinoma cell lines was isolated using Quick-RNA™ MiniPrep (Zymo Research, Irvine, CA) or miRNeasy® Mini Kit (Qiagen, Valencia, CA) spin columns per manufacturer instructions. Total RNA was eluted from columns with DEPC H₂O. Isolated RNA was DNase (Roche, Mannheim, Germany) treated per manufacturer instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Probe-based assays for real-time reverse transcription-polymerase chain reaction (RT-PCR) were designed using Primer Express software (Life Technologies-Applied Biosystems, Carlsbad, CA). Primer and probe sequences, accession number, and accompanying information for all assays are detailed in Table 12.1. Design and development of all quantitative real-time RT-PCR assays was achieved by collaboration with Dr. Gregory Shipley, The University of Texas at Houston - Medical School, Houston, Texas. 100ng RNA aliquots were reverse transcribed in quadruplicates, including a no reverse transcriptase control, with 300nM assay-specific reverse primer, 4mM MgCl₂, 500μM dNTPs, and 10 units of MMLV Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 50°C for 30 minutes, followed by 72°C for 5 minutes. 40μl of PCR mix containing 1X PCR buffer, 300nM specific forward and reverse primers, 4mM MgCl₂, Taq DNA polymerase, and 100nM fluorogenic probe were added to each 10μl RT reaction. Amplification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Transcript levels were determined through comparison to a standard curve generated from the PCR amplification of template dilutions covering a 5-log range. Final transcript values were normalized to 18S ribosomal RNA (rRNA), dilution of 1/500 or 1/200 and presented as mean transcript levels expressed as %18SrRNA (molecules of transcript/molecules of 18S rRNA). Primer and probe sequences and accession numbers of assays developed and used in this dissertation are indicated in Table 3.1.

Immunofluorescence

Normal and Carcinoma Tissues

Cryosections, 4μm in thickness, of normal colon and endometrium and endometrial carcinomas were sectioned by MDA-GCTR-TB. Room temperature warmed sections were fixed

Table 3.1. Real-time quantitative RT-PCR assays

	<i>Gene Symbol</i>	Taqman Primers and Probe	Accession Number
5'-Nucleotidase	<i>CD73</i>	FP: 1447+GACAGAGTAGTCAAATTAGATG RP: 1511-TGAGAGGGTCATAACTGG P: 1471+TCTTTGCACCAAGTGTCTCGAGTGC	NM_002526
Adenosine Deaminase	<i>ADA</i>	FP: 264+CTGCTGAACGTCATTGG RP: 340-GCAGGCATGTAGTAGTC P: 281+CATGGACAAGCCGCTCACCC	NM_000022
Adenosine Receptor 1	<i>A1R</i>	FP: 1147+GCTGGCTGCCTTTGCAC RP: 1215-GGATGCTGGGCTTGTGG P: 1165+TCCTCAACTGCATCACCTCTTCTGC	NM_000674
Adenosine Receptor 2A	<i>A2AR</i>	FP: 838+ATGCTGGGTGTCTATTTGCG RP: 902-TGGCTCTCCATCTGCTTCAG P: 865+CTGGCGGCGCGACGACA	NM_000675
Adenosine Receptor 2B	<i>A2BR</i>	FP: 977+CACTGAGCTGATGGACCACTC RP: 1040-CAGTGACTTGGCTGCATGG P: 1018-TCCCGCTGGAGGGTGGTCCT	NM_000676
Adenosine Receptor 3	<i>A3R</i>	FP: 708+CCCTACAGACGGATCTTGCTG RP: 777-TGTTGGGCATCTTGCCTTC P: 734+CCTGTCCCTGTGGAGTTCCCCT	NM_000677
Equilibrative Nucleoside Transporter 1	<i>ENT1</i>	FP: 1413+CCAGCCGTGACTGTTGAG RP: 1489-CAGGACACAGGAATGAAGTAAC P: 1438+FAM CCAGCATCGCAGGCAGCAGC	NM_001078177
Osteopontin	<i>SPP1</i>	FP: 619+GGAAGTGGAGGTCAAATCTAAGAAG RP: 693-GGTGATGTCCTCGTCTGTAG P: 646+CGCAGACCTGACATCCAGTACCCT	NM_001040058
Purinergic Receptor P2Y	<i>P2RY2</i>	FP: 1212+GTGGTGCTGGCTGTCTTC RP: 1286-CGAGCGGAAGGAGTAGTAG P: 1232+CCTCTGCTTCCTGCCATTCCACG	NM_176072

Abbreviations: FP, Forward Primer; RP, Reverse Primer; P, Probe

in 4% paraformaldehyde for 1 hour at 4°C and washed with 1X phosphate buffered saline (PBS). Tissues sections were blocked with Background Sniper (Biocare Medical, Concord, CA) for 1 hour at room temperature and incubated with anti-human CD73, 1:100 (Hycult Biotech, Plymouth, PA) and wide spectrum cytokeratin, 1:500 (Abcam, Cambridge, MA) at 4°C overnight. Following incubation, tissues were washed with 1X PBS and incubated with fluorochrome-conjugated secondary antibodies, anti-mouse Alexa Fluor® 594 and anti-rabbit Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) at room temperature for 1 hour. Tissue sections were washed with 1X PBS, incubated with DAPI Nucleic Acid Stain (Invitrogen, Carlsbad, CA), and mounted with DakoCytomation Antifade Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Primary and secondary antibodies were diluted in Background Sniper. For spectral bleed-through artifact controls, tissue sections were incubated with each fluorochrome separately. Autofluorescence of tissue sections was determined by substituting primary antibodies with 1X PBS. Images were captured using a IX71 Olympus microscope (Center Valley, PA) and Image-Pro MC 6.1 imaging software. CD73 fluorescence intensity was measured in four 20X field images using NIH ImageJ software. Data is represented as mean fold increase of CD73 intensity in epithelial/carcinoma cells relative to surrounding stroma cells/connective tissue.

Carcinoma Cell Lines

Colon and endometrial carcinoma cell lines were grown to 70% confluency on 1- 2- or 4-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY). After fixation with 4% paraformaldehyde for 1 hour at 4°C, cells were washed with 1X PBS, and permeabilized with 0.1% triton X100 (Sigma-Aldrich, St. Louis, MO) for 20 minutes, room temperature. Cells were blocked with Background Sniper (Biocare Medical, Concord, CA) for 1 hour at room temperature and incubated with respected primary antibodies at 4°C overnight. Following incubation, cells were washed with 1X PBS and incubated with fluorochrome-conjugated

secondary antibodies, anti-mouse Alexa Fluor® 594 and anti-rabbit Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) at room temperature for 1 hour. Cells were washed with 1X PBS, incubated with DAPI Nucleic Acid Stain (Invitrogen, Carlsbad, CA), and mounted with DakoCytomation Antifade Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Primary and secondary antibodies were diluted in Background Sniper. For spectral bleed-through artifact controls, tissue sections were incubated with each fluorochrome separately. Autofluorescence was determined by substituting primary antibodies with 1X PBS. Images were captured using a IX71 Olympus microscope (Center Valley, PA) and Image-Pro MC 6.1 imaging software.

Immunofluorescence – Antibodies

Primary antibodies and their working dilutions are as follows. CD73, 1:100, Hycult Biotech, Plymouth Meeting, PA; E-cadherin, 1:500, BD Biosciences, San Jose, CA; Na⁺K⁺ ATPase, 1:200, Epitomics, Burlingame, CA; Pan-cytokeratin, 1:200, Abcam, Cambridge, MA; VASP, 1:100, Cell Signaling, Danvers, MA; Vimentin, 1:200, Cell Signaling, Danvers, MA.

Immunoblot

Carcinoma cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, 150mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na deoxycholate) (Upstate, Temecula, CA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany). 20µg of isolated protein was combined with 4X loading buffer and β-mercaptoethanol and boiled for 5 minutes at 95°C. 8% or 10% polyacrylamide gels were used for the protein samples. Protein samples were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA), blocked overnight with 5% blotto-TBS/Tween, and probed with the following antibodies: Adenosine receptor 2B (A2BR), 1:500, Millipore, Billerica, MA; β-actin, 1:5,000, Sigma, St

Louis, MO; E-cadherin, 1:1000, BD Biosciences, San Jose, CA; HIF-1 α , 1:500, BD Biosciences, San Jose, CA; phospho-Smad2 (p-Smad2 Ser465/467), 1:500, Cell Signaling, Danvers, MA; Smad2, 1:500, Cell Signaling, Danvers, MA; vinculin, 1:500, Sigma, St Louis, MO.

***In Vivo* Ruthenium Red Paracellular Permeability**

Vaginal Smears

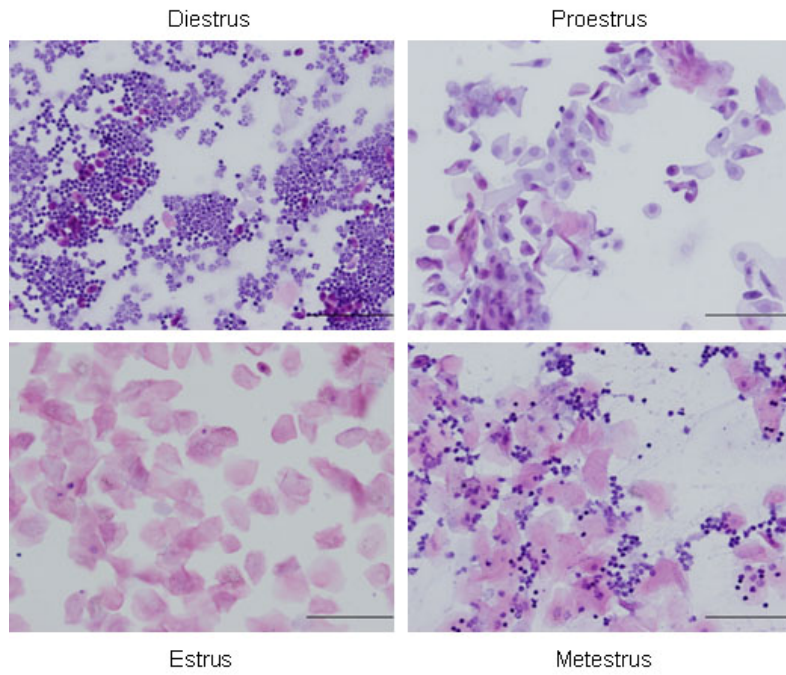
10-14 week-old C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice were maintained in a dark:light cycle of 12:12 hours. Phase of estrous was determined daily by vaginal smears. Mice were anesthetized by isoflurane prior to collecting vaginal smears. Cells were flushed from the vaginal lining by introducing 20ul of sterile 1X phosphate buffered saline (PBS) into the vaginal opening. Recovered cell suspensions were smeared on glass microscope slides and fixed with 70% ethanol. After re-hydrating in absolute alcohol, slides were progressed through an alcohol gradient of 90% and 70%, rinsed with dH₂O, and stained with hematoxylin for 1 minute. Slides were rinsed with dH₂O, stained with eosin for 30 seconds, and progressed through an alcohol gradient of 70%, 90%, and 100%. Cell morphology and phase determination was performed using a BX41 Olympus microscope (Center Valley, PA). To minimize the incidence of translational or missed stages, vaginal smears were performed daily at 09:00. Representative images of vaginal smears are shown in Figure 3.1.

Ruthenium Red Uterine Perfusions

Estrous cycles of 10-14 week-old C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice were synchronized and stage of cycle determined by vaginal smear. Proestrus staged mice were individually placed in a modular incubator chamber (Billups-Rothenberg, Delmar, CA) and exposed to room air or hypoxia (7% O₂) for 1, 2, or 4 hours. Following allotted time-of-exposure, mice were anesthetized

Figure 3.1. Representative images of diestrus, proestrus, estrus, and metestrus vaginal smears. 10-14 week-old C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice were maintained in a dark:light cycle of 12:12 hours. Vaginal smears were taken on consecutive days. Diestrus smears consist mainly of leucocytes with variable numbers of epithelial cells. Proestrus smears are characterized by rounded, usually nucleated, epithelial cells, generally in low to moderate numbers. Estrus smears consist entirely of cornified epithelial cells in high numbers, which usually form sheets of cells. Metestrus smears are characterized by equal numbers of cornified epithelial cells and leucocytes. Image magnification 20X.

Figure 3.1



by tribromoethanol (Avertin) dosed at 0.2ml/10g. With the peritoneal cavity exposed, the left uterine horn was initially perfused-fixed with 0.2ml 0.2% ruthenium red (Sigma-Aldrich, St Louis, MO) and 2% glutaraldehyde (Sigma-Aldrich, St Louis, MO) in 0.1M sodium cacodylate buffer. Following surgical closure of the endocervical region, the uterine horn was filled with 0.1ml 0.2% ruthenium red and 2% glutaraldehyde in 0.1M sodium cacodylate buffer. Mice exposed to hypoxia were returned to the modular incubator chambers for 10 minutes. Uteri were then removed and re-perfused-fixed, in order, with 0.7ml 0.2% ruthenium red and 2% glutaraldehyde in 0.1M sodium cacodylate buffer and 0.2ml 0.2% ruthenium red in 2% O_5O_4 in 0.1M sodium cacodylate buffer. In additional studies, $CD73^{-/-}$ mice were injected IP with 5'-N-ethylcarboxamidoadenosine (NECA) 0.1mg/kg or 0.9% NaCl containing an equal volume of NECA vehicle, dimethylsulfoxide (DMSO) 0.1ml/20g (DMSO content, 0.006%) prior to being exposed to hypoxia for 4 hours. This protocol was in accordance with the National Institute of Health (NIH) guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Houston. This technique was modified and adapted from Molitons, BA., Falk, SA., and Dahl, RH., Ischemia-induced loss of epithelial polarity role of the tight junction, JCI 1989;84:1334-1339 (229).

Ruthenium Red Uterine Perfusions – Transmission Electron Microscopy

Perfused-fixed uteri were longitudinally cut into 1mm sections, washed in 0.1M sodium cacodylate buffer, dehydrated in graded ethanol solutions, and embedded in LX-112 medium. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (model JEM 1010, JEOL Inc., Peabody, MA). Digital images were acquired using an AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA). Ruthenium red paracellular permeability was quantified by assessing

approximately 200 consecutive paracellular spaces per uterine section. Percent ruthenium red permeability was determined by the ratio of dye positive to total paracellular spaces assessed.

Transforming Growth Factor- β (TGF- β) Studies

Transepithelial Resistance (TER)

HEC-1A cells were plated at a density of 1×10^5 cells and grown to confluency on transwell-collagen coated permeable inserts, 0.4 μ m pore size, 12 well plates (Corning, Corning, NY). Medium was changed every 24 hours until a stable monolayer was attained. Following 4 days post-confluency, HEC-1A monolayers were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). The Opti-MEM® in the inserts and lower chambers was replaced with Opti-MEM® alone or containing human recombinant 2.5ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN) or equal vehicle, 4mM HCl with 1mg/mL of bovine serum albumin (BSA) (Sigma, St. Louis, MO). The electrical potential difference across the monolayer was measured using a two-electrode EVOM voltohmmeter (World Precision Instruments Inc., New Haven, CT). Resistance measurements were taken every 24, 48, 72, 96, and 120 hours following the initial treatment of the cells. Opti-MEM® with or without TGF- β 1 or BSA/HCl, was replaced every 24 hours. Data is expressed as percent increased TER from mean baseline.

CD73 Regulation by TGF- β 1 – Quantitative RT-PCR Time-Course

Endometrial carcinoma cells, HEC-1A and Ishikawa, were plated in 6-well plates at a density of 1×10^5 cells/well in respective base mediums containing 10% FBS. After reaching 70% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were treated with human recombinant 2.5ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN) or equal vehicle, 4mM HCl with 1mg/mL of

bovine serum albumin (BSA) (Sigma, St. Louis, MO) in Opti-MEM® for 24, 48, 72, 96, and 120 hours. In separate studies, cells were treated for 12, 24, 48, and 72 hours. RNA was isolated in experimental replicates of 4 from each time-course. Isolated RNA was DNase (Roche, Mannheim, Germany) treated per manufacturer instructions. Real-time quantitative RT-PCR was performed at The University of Texas Quantitative Genomics Core Laboratory. In separate studies, cells were co-incubated with 1µM of Smad3 inhibitor, SIS3 (Calbiochem, San Diego, CA).

CD73 Regulation by TGF-β1 – Immunofluorescence Time-Course

HEC-1A cells were plated at a density of 1×10^4 cells/well in 2-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY). After reaching 70% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were treated with human recombinant 2.5ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) or equal vehicle, 4mM HCl with 1mg/mL of bovine serum albumin (BSA) (Sigma, St. Louis, MO) in Opti-MEM® for 24, 48, and 72 hours. In separate studies, cells were co-incubated with 1µM of Smad3 inhibitor, SIS3 (Calbiochem, San Diego, CA).

CD73 Regulation by TGF-β1 – Immunoblot Time-Course

HEC-1A cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and allowed to reach 70% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were treated with human recombinant 2.5ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) or equal vehicle, 4mM HCl with 1mg/mL of bovine serum albumin (BSA) (Sigma, St. Louis, MO) in Opti-MEM® for 24, 48, 72, 96, and 120 hours. Protein lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (50mM

Tris, 150mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na deoxycholate) (Upstate, Temecula, CA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany). Densitometry was determine from 2 separate studies using NIH ImageJ software.

CD73 and A2BR Induced Expression by Hypoxia

Endometrial carcinoma cells, HEC-1A and HEC-1B, and cervical carcinoma cells, WISH-HeLa, were plated in 6-well plates at a density of 1×10^5 cells/well in respective base mediums containing 10% FBS. After reaching 90% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were placed in normoxic or hypoxic (1% O₂, 5% CO₂) conditions. RNA was isolated at 4, 6, 12, and 24 hours following hypoxic or normoxic exposure. Isolated RNA was DNase (Roche, Mannheim, Germany) treated per manufacturer instructions. Real-time quantitative RT-PCR was performed at The University of Texas Quantitative Genomics Core Laboratory. In separate studies, cells were co-incubated with 1μM of Smad3 inhibitor, SIS3 (Calbiochem, San Diego, CA).

Increased Steady-State Levels of HIF-1α by Hypoxia

HEC-1A, HEC-1B, and WISH-HeLa cells were plated at a density of 1×10^6 cells/plate in respective base mediums containing 10% FBS. After reaching 90% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were placed in normoxic or hypoxic (1% O₂, 5% CO₂) conditions. Protein lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, 150mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na deoxycholate) (Upstate, Temecula, CA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany) at time points of 4, 6, 12, and 24 hours.

CD73 Catalytic Studies

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Protein lysates from normal endometrium, endometrial carcinomas, and endometrial carcinoma cell lines were freshly prepared using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, 150mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na deoxycholate) (Upstate, Temecula, CA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany). For determining specific activity for CD73, 10 μ g of protein lysates were incubated with 400 μ M 5'AMP (Sigma, St. Louis, MO) for 15 minutes, 37°C in the presence of 10 μ M deoxyconformycin in HEPES buffer, with or without 1mM of α , β - methylenediphosphate (AOPCP) (Sigma, St. Louis, MO). Heat-inactivated protein lysates were used as negative controls. Reactions were terminated with 5 minute incubation at 95°C. Reactions were analyzed for adenosine production using reverse-phase (C18) high performance liquid chromatography. CD73 specific activity was expressed as nmole production per min per mg of protein (nmole/min/mg).

CD73 Enzyme Histochemistry

Normal Colon and Endometrium and Endometrial Carcinomas

Cryosections, 15 μ m in thickness (230), of normal endometrium and endometrial carcinomas were sectioned by MDA-GCTR-TB. Room temperature warmed sections were fixed in 0.05M cacodylate-buffered 4% paraformaldehyde for 15 minutes and washed in 0.05M cacodylate buffer containing 0.25M sucrose. Sections were incubated for 1.5 hours at room temperature, in a reaction solution containing 1mM 5'AMP, 2mM Pb(NO₃)₂, 5mM MnCl₂, 0.25M sucrose, and 50mM tris-malate pH 7.4. 5mM tetramisole hydrochloride (levamisole) was included to block enzymatic activity of non-specific phosphatases. Further specificity for CD73 activity was demonstrated in selected sections by including 1mM α , β -methylene diphosphate (AoPCP). For negative controls, 5'AMP was omitted. Following incubation, sections were washed in dH₂O

and immersed in 2% (NH₄)₂S for 10 seconds. Sections were mounted with DAPI containing permount. This technique was modified from Wachstein, M. and Meisel, E., Histochemistry of hepatic phosphatase at a physiological pH with special reference to the demonstration of bile canaliculi, Am. J. Clin. Pathol. 1957;27:13-23 (231). Images were captured using a BX41 Olympus microscope (Center Valley, PA) and Image-Pro MC 6.1 imaging software.

Colon and Endometrial Carcinoma Cell Lines

Colon and endometrial carcinoma cell lines were grown to 70% or 90% confluency on 2-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY). Fixation, reaction solution incubation, and phosphate precipitation methods were identical to those described for the cryosections.

CD73 Enzyme Histochemistry – Transmission Electron Microscopy

Enzyme histochemistry was performed on HEC-1A cells as described. Cells were fixed in 2% glutaraldehyde (Sigma, St. Louis) for 1 hour at 4°C, washed in 0.1M sodium cacodylate buffer, dehydrated in graded ethanol solutions, and embedded in LX-112 medium. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (model JEM 1010, JEOL Inc., Peabody, MA). Digital images were acquired using an AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

WISH-HeLa Time-Course

WISH-HeLa Time-Course - Histology

WISH-HeLa cells were plated at a density of 1x10⁴ cells/well in 2-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY) and observed for cell confluencies of 50%, 70%, 90%, 100% and 2- and 4-day post-confluency. Chamber slides were fixed at 4°C in 4% paraformaldehyde for 1 hour, re-hydrated in absolute alcohol, moved through an alcohol gradient

of 90% and 70%, rinsed with dH₂O, and stained with hematoxylin for 1 minute. Chamber slides were rinsed with dH₂O, stained with eosin for 30 seconds, and progressed through an alcohol gradient of 70%, 90%, and 100%. Images were captured using a BX41 Olympus microscope (Center Valley, PA) and Image-Pro MC 6.1 imaging software. WISH-HeLa cell area and perimeter was measured using The National Institute of Health (NIH) analysis software, ImageJ (NIH, Bethesda, MD, <http://rsb.info.nih.gov/nih-image>). Eight to ten images of a 40X field were measured per percent/post-confluency timepoint.

WISH-HeLa Time-Course - Quantitative RT-PCR

WISH-HeLa cells were plated in 6-well plates at a density of 1×10^5 cells/well and observed for growth equal to percent cell confluencies of 70%, 90%, 100% and 2- and 4-day post-confluency. RNA was isolated in 4 experimental replicates from each confluency stage. Isolated RNA was DNase (Roche, Mannheim, Germany) treated per manufacturer instructions. Real-time quantitative RT-PCR was performed at The University of Texas Quantitative Genomics Core Laboratory.

WISH-HeLa Time-Course - Immunofluorescence

WISH-HeLa cells were plated at a density of 1×10^5 cells/chamber in 1-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY) and observed for growth equal to percent cell confluencies of 70%, 90%, 100% and 2- and 4-day post-confluency. Chamber slides were fixed at 4°C in 4% paraformaldehyde for 1 hour and advanced through immunostaining methods.

Small Interfering RNA (siRNA) – CD73 Silencing

CD73 Silencing - Synthetic 21-mer siRNAs

Three individual synthetic 21-mer siRNAs for CD73 were custom designed (Sigma-Aldrich, St. Louis, MO). siRNA sequences were adapted from Mikhailov et al., CD73 participates in cellular multiresistance program and protects against TRAIL-induced apoptosis, *J Immunol* 2008;181:464-475 (232). Individual CD73 siRNA oligonucleotide sequences are detailed in Table 3.2

CD73 Silencing – Optimization

HEC-1A cells were plated in 6-well plates at a density of 1×10^5 cells/well in McCoy's 5A medium containing 10% FBS. After reaching 50% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were transfected with varying concentrations, 10nM, 25nM, and 100nM, of individual CD73 siRNAs or the non-targeting siRNA or transfected with a combination mixture, a 1:1:1 ratio, of individual CD73 siRNAs. Individual and combination concentrations were 10nM, 25nM, and 100nM. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used as the cationic lipid carrier. RNA was isolated in experimental replicates of 3 from each group 24 hours after siRNA transfection. Effectiveness of CD73 silencing was determined by real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed at The University of Texas Quantitative Genomics Core Laboratory (Figure 3.2). For CD73 silencing studies in this dissertation, 25nM of CD73 siRNA 1247 was used.

CD73 Silencing – Quantitative RT-PCR 1247 CD73 siRNA

HEC-1A cells were plated in 6-well plates at a density of 1×10^5 cells/well in McCoy's 5A medium containing 10% FBS. After reaching 50% confluency, cells were serum starved for 24

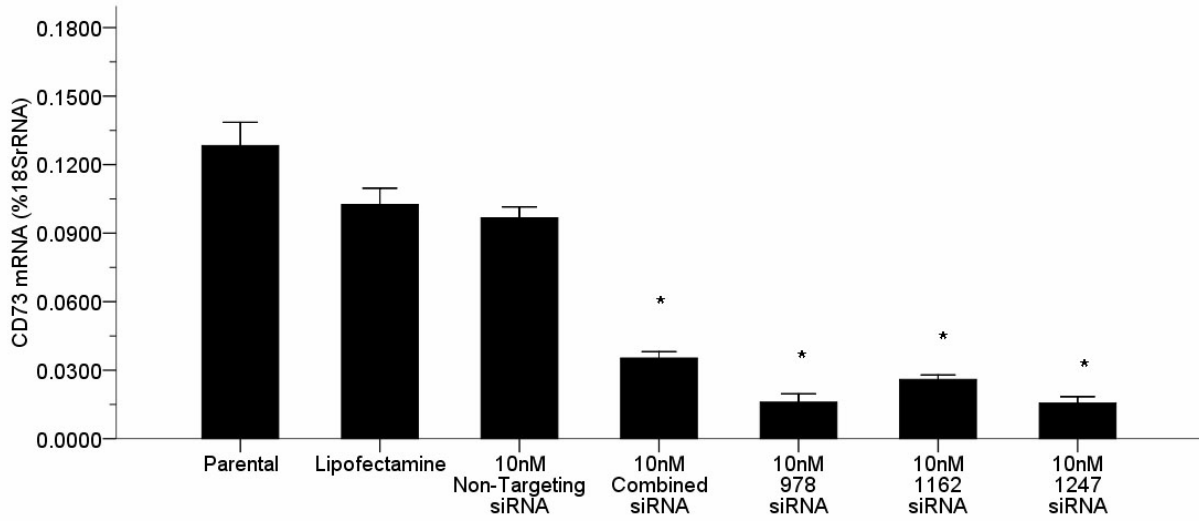
Table 3.2. Individual CD73 and non-targeting siRNA oligonucleotide sequences

CD73 siRNA	Oligonucleotide Sequence
978	5'-ACAGCAGCAUJCCUGAAGATT-3'
1162	5'-CCUGAGACACACGGAUGAATT-3'
1247	5'-CGCAACAAUGGCACAAUUATT-3'
Non-Targeting	5'-GAUCAUACGUGCGAUCAGATT-3'

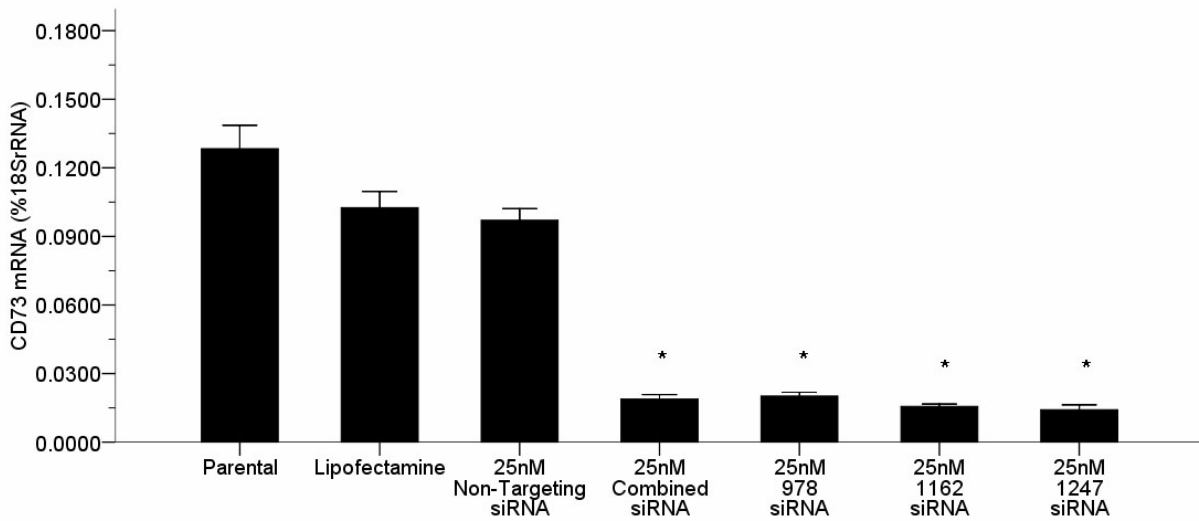
Figure 3.2. Optimization of CD73 siRNAs. Endometrial carcinoma cells, HEC-1A, were transiently transfected with individual CD73 siRNAs or the non-targeting siRNA or transfected with a combination mixture, a 1:1:1 ratio, of individual CD73 siRNAs. Individual and combination concentrations used were 10nM, 25nM, 100nM. *CD73* expression was determined 24 hours post-transfection by qRT-PCR. (A) 10nM, (B) 25nM, (C), 100nM concentrations of individual and combined CD73 siRNA oligonucleotides. With 100nM concentrations, a small change in *CD73* expression is additionally found with the non-targeting control, compared to the lipofectamine control. This suggest that concentrations of 100nM may be leading to off-target effects. Error bars represent ± 1 SE. (* $p < 0.05$)

Figure 3.2

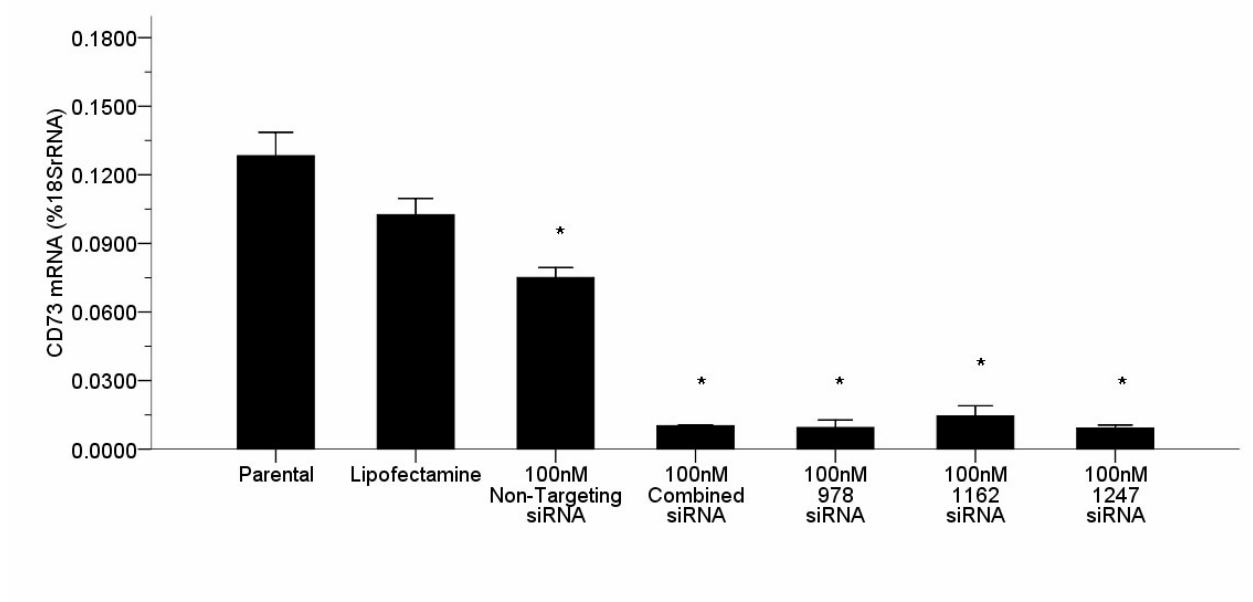
A



B



C



hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA) and transfected with 25nM of CD73 siRNA 1247, non-targeting siRNA, or Lipofectamine™ 2000 alone. RNA was isolated in 3 experimental replicates 24 hours post-transfection. CD73 silencing with transient transfection was determined by quantitative RT-PCR. Real-time quantitative RT-PCR was performed at The University of Texas Quantitative Genomics Core Laboratory.

CD73 Silencing - Proliferation

HEC-1A cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium containing 10% FBS. After reaching 50% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA) and transfected with 25nM of CD73 siRNA 1247, non-targeting siRNA, or Lipofectamine™ 2000 alone. Following 24 hours, cells were trypsinized and re-plated in 12-well plates at a density of 1×10^5 cells/well (Day 0). Effects of CD73 silencing on HEC-1A proliferation were determined by manual cell counting for 5 consecutive days. Trypan Blue (Sigma-Aldrich, St. Louis, MO) was included in counting suspensions to concurrently observe cell viability. Three experimental replicates were performed for each group.

CD73 Silencing – Immunofluorescence

HEC-1A cells were plated at a density of 1×10^4 cells/well in 2-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY), observed for growth equal to 50%, and serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were transfected with 25nM of CD73 siRNA 1247, non-targeting siRNA, or Lipofectamine™ 2000 alone. Medium was replaced 24 hours post-transfection with OptiMEM® Reduced Serum Medium. Chamber slides of 48 or 72 hours post-transfection were fixed at 4°C in 4% paraformaldehyde for 1 hour and advanced through immunostaining methods.

Flow cytometry – Annexin V

HEC-1A and HEC-1B cells plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in normoxic conditions or 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point). Annexin-V staining (Roche, Mannheim, Germany) was performed per manufacturer instructions. Samples were analysed by flow cytometry at the University of Texas MD Anderson Cancer Center Flow Cytometry & Cellular Imaging Core.

Modified and Unmodified Boyden Chambers – Migration and Invasion

Migration and invasion studies were performed using the modified, non-coated membrane, and unmodified, Matrigel™ (BD Bioscience, Bedford, MA) coated membrane, two-chamber system described by Albini, A. et al., A rapid in vitro assay for quantitating the invasive potential of tumor cells, *Cancer Res.* 1987;47:3239-3245 (233) and Tumor and endothelial cell invasion of basement membranes: the matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms, *Pathol Oncol Res.* 1998;4(3):230-241 (234). Membranes of modified and unmodified systems consisted of an 8µm diameter pore size and 6 pores/cm² pore density (BD Bioscience, Bedford, MA). Cells were plated at a density of 3×10^5 cells/insert in OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). OptiMEM® containing 5% FBS was used as a chemoattractant. Chambers were incubated at 37°C in normoxic conditions or 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point). Following incubation cells were removed from the upper-chamber, and lower-chamber membrane-bound cells were fixed with 100% methanol. Cells were stained by incubating membranes in eosin, 30 seconds, and hematoxylin, 5 minutes. Six or 9 10X images per membrane

were captured using a BX41 Olympus microscope (Center Valley, PA) and Image-Pro MC 6.1 imaging software. Quantification was performed by counting the number of cells present in a 10X field. Cell counting was assisted by using The National Institute of Health (NIH) analysis software, ImageJ (NIH, Bethesda, MD, <http://rsb.info.nih.gov/nih-image>). Data are expressed as mean number of cells that have migrated or invaded the membrane in a 10X field. Experimental replicates of 3, 4, or 6 were performed for each group. Results were validated by a second series of independent experiments.

Migration and Invasion – Normoxia-Hypoxia

HEC-1A and HEC-1B cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA) and removed from plates using 0.25% trypsin (Invitrogen, Carlsbad, CA). Cells were re-suspended in OptiMEM® containing 50uM of 5'adenosine monophosphate (5'AMP) (Sigma-Aldrich, St. Louis, MO) and plated at a density of 3×10^5 cells/insert. Inserts were incubated at 37°C in normoxic conditions or 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point).

Migration and Invasion – CD73 siRNA

HEC-1A and HEC-1B cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 50% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Following serum starvation, cells were transfected with 25nM of CD73 siRNA 1247, non-targeting siRNA, or Lipofectatine™ 2000 alone. Medium was replaced 24 hours post-transfection with OptiMEM® Reduced Serum

Medium. Cells were removed from plates 55-60 hours post-transfection, re-suspended in OptiMEM® containing 50uM of 5'adenosine monophosphate (5'AMP) (Sigma-Aldrich, St. Louis, MO) and plated at a density of 3×10^5 cells/insert. Inserts were incubated at 37°C 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point).

Migration and Invasion – CD73 Catalytic Inhibition

HEC-1A and HEC-1B cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to trypsinization, selected groups were pre-incubated with 10uM or 100uM α,β -methylene diphosphate (AoPCP) (Sigma-Aldrich, St. Louis, MO) for 1 hour. Cells were re-suspended in OptiMEM® containing 50uM of 5'adenosine monophosphate (5'AMP) (Sigma-Aldrich, St. Louis, MO) with or without 10uM or 100uM AoPCP and plated at a density of 3×10^5 cells/insert. Inserts were incubated in normoxic conditions or 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point). For determining receptor-mediated response, 10 μ M 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO) was included in cell suspensions of selected 5'AMP/AoPCP groups.

Migration and Invasion – Adenosine Receptor Antagonism

HEC-1A and HEC-1B cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to trypsinization, selected groups were pre-incubated with 1uM of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)

(Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Cells were re-suspended in OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO); 10µM 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); 10µM NECA and 1µM DPCPX; 10µM NECA and 1µM MRS1754. Cells were plated at a density of 3x10⁵ cells/insert and inserts incubated at 37°C, 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point) or 36 hours (invasion end-point).

Migration and Invasion – Vinculin Neutralization

HEC-1A cells were plated in 100mm plates at a density of 1x10⁶ cells/plate in McCoy's 5A medium or Minimum Essential Medium (MEM) containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to trypsinization, selected groups were pre-incubated with 10µM rabbit IgG or 10µM vinculin for 20 minutes. Cells were additionally pre-incubated with 1µM of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Cells were re-suspended in OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO); 10µM 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); 10µM NECA and 1µM DPCPX; 10µM NECA and 1µM MRS1754. Cells were additionally re-suspended with 10µM rabbit IgG or 10µM vinculin. Cells

were plated at a density of 3×10^5 cells/insert and inserts incubated at 37°C, 1% O₂, 5% CO₂ (hypoxia) for 24 hours (migration end-point).

Adenosine Receptor Antagonism –Macromolecule Paracellular Permeability

HEC-1A cells were grown on polycarbonate permeable inserts (0.4- μ m pores, 24-mm diameter; Costar, Cambridge, MA). At 2 days post-confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Selected inserts were pre-incubated with 1 μ M of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Medium was removed and replaced with OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO); 10 μ M 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); 10 μ M NECA and 1 μ M DPCPX; 10 μ M NECA and 1 μ M MRS1754. Inserts were incubated at 37°C, 1% O₂, 5% CO₂ (hypoxia) for 36 hours. Inserts were washed with Hank's balanced salt solution (HBSS). At time 0, 1.5mL of HBSS was added to the lower wells, and 0.5mL of HBSS containing 3.5 μ M fluorescein isothiocyanate (FITC)-labeled 40-kDa dextran (NANCOS, New York, NY) was added to the inserts. At 10, 20, 30, and 40 minutes after addition of FITC-labeled dextran, 100- μ l samples were taken from the lower wells. Sample volumes were replaced with equal volumes of HBSS. The fluorescence intensity of each sample was measured at an excitation wavelength of 485nm and an emission wavelength of 530nm using a FLUOstar Omega Microplate Reader (BMG Labtech, Cary, NC).

Adenosine Receptor Antagonism – Immunofluorescence

HEC-1A cells were plated at a density of 1×10^4 cells/well in 2 or 4-well chamber slides (Lab-Tek™, Nalge Nunc International, Rochester, NY). After reaching 90% confluency, cells were serum starved for 24 hours using Opti-MEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to hypoxia incubation, selected chamber slides were pre-incubated with $1 \mu\text{M}$ of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Medium was removed and replaced with OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO); $10 \mu\text{M}$ 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); $10 \mu\text{M}$ NECA and $1 \mu\text{M}$ DPCPX; $10 \mu\text{M}$ NECA and $1 \mu\text{M}$ MRS1754. Cells were incubated at 37°C , 1% O_2 , 5% CO_2 (hypoxia) for 36 hours.

Adenosine Receptor Antagonism – Immunoblots

HEC-1A cells were plated in 100mm plates at a density of 1×10^6 cells/plate in McCoy's 5A medium containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to hypoxia incubation, selected plates were pre-incubated with $1 \mu\text{M}$ of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Medium was removed and replaced with OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis,

MO); 10 μ M 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); 10 μ M NECA and 1 μ M DPCPX; 10 μ M NECA and 1 μ M MRS1754. Cells were incubated at 37°C, 1% O₂, 5% CO₂ (hypoxia) for 36 hours. Protein lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, 150mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na deoxycholate) (Upstate, Temecula, CA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany).

Adenosine Receptor Antagonism – Electron Microscopy

HEC-1A cells were plated in 100mm plates at a density of 1x10⁶ cells/plate in McCoy's 5A medium containing 10% FBS and allowed to reach 90% confluency. Cells were serum starved for 24 hours using OptiMEM® Reduced Serum Medium, GlutaMax™ (Invitrogen, Carlsbad, CA). Prior to hypoxia incubation, selected plates were pre-incubated with 1 μ M of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, St. Louis, MO), an adenosine A1 receptor (A1R) antagonist, or *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (Tocris Bioscience, Ellisville, MO), an adenosine 2B receptor (A2BR) antagonist for 20 minutes. Medium was removed and replaced with OptiMEM® or OptiMEM® containing the following: dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO); 10 μ M 5'-N-ethylcarboxamidoadenosine (NECA) (Tocris Bioscience, Ellisville, MO); 10 μ M NECA and 1 μ M DPCPX; 10 μ M NECA and 1 μ M MRS1754. Cells were incubated at 37°C, 1% O₂, 5% CO₂ (hypoxia) for 36 hours. Cells were fixed in a solution containing 0.2% ruthenium red and 2% glutaraldehyde in 0.1M sodium cacodylate buffer and 0.2ml 0.2% ruthenium red in 2% O₅O₄ in 0.1M sodium cacodylate buffer. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (model JEM 1010, JEOL Inc., Peabody, MA). Digital images were acquired using an AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

CHAPTER FOUR

Statement of Objectives

Objective

The preliminary objective of this dissertation was to identify which purinergic members central to adenosine's extracellular generation and regulation are dysregulated in EC. Following the identification of CD73's down-regulation in moderately- and poorly-differentiated, invasive, and metastatic EC, the primary objective was to establish the role of CD73-generated adenosine to induce barrier function in normal epithelial cells of the endometrium, and to identify the cell-cell adhesion events or proteins that adenosine's activation of A2BR regulates in carcinoma cells. Our primary **hypothesis** is that the purinergic pathway, especially CD73-generated adenosine, is important for maintaining barrier function in epithelial cells of the endometrium via the regulation of cell-cell adhesions. In addition, we **hypothesize** that adenosine's activation of A2BR inhibits the invasiveness of EC cells and may be a molecular determinant of therapy sensitivity. To address these hypotheses, we have used a variety of different experimental approaches including *in vivo* functional and structural experiments using knock-out mice, *in vitro* functional and structural studies using established cell lines, and expression studies using tissues derived from normal human endometrium and well-characterized endometrial carcinomas. The following specific aims were proposed and completed:

Specific Aim 1. Identify the components of purinergic signaling that are dysregulated in endometrial carcinoma. Probe-based real-time quantitative RT-PCR assays for high-throughput analysis were designed for select members of the purinergic pathway and assessed in normal endometrium and endometrial carcinomas.

Specific Aim 2. Demonstrate that CD73-generated adenosine serves a critical barrier protective role in epithelial cells of the endometrium and that CD73 expression is induced by known stimuli of barrier function.

Sub-aim 2A. An *in vivo* ruthenium red paracellular permeability assay was developed to determine loss of endometrial epithelial barrier function in conditions of hypoxic stress in mice deficient for CD73 or A2BR. Paracellular flux of the electron dense ruthenium red was quantified in wild-type mice, CD73 deficient mice, and A2BR deficient mice.

Sub-aim 2B. Investigate the ability of *in vitro* TGF- β 1 and hypoxia's stabilization of HIF-1 α to induce CD73 and/or A2BR expression.

Specific Aim 3. Demonstrate that CD73's pattern of expression and catalytic activity are consistent with the proposed role of CD73 in the maintenance of the epithelial phenotype.

CD73 expression and specific activity were assessed by reverse phase high performance liquid chromatography (RP-HPLC), immunofluorescence, and enzyme histochemistry. Change in CD73 expression with the transitioning of cellular phenotypes was assessed in WISH-HeLa cells.

Specific Aim 4. Show that CD73-generated adenosine and adenosine's activation of A2BR inhibits the migration and invasion of endometrial carcinoma cells. *In vitro* migration and

invasion studies were performed using modified and unmodified two-chamber systems. siRNA and commercially available pharmacological agents were used to manipulate CD73 activity and A2BR activation.

Specific Aim 5. Identify the events or proteins of cell-cell adhesion that are regulated by adenosine's activation of A2BR. This specific aim assesses the events and proteins of cell-cell adhesions that are affected by the manipulation of A2BR activation.

CHAPTER FIVE

Results

Specific Aim 1. Identify the components of purinergic signaling that are dysregulated in endometrial carcinoma.

Introduction

A hallmark of cancer cell biology is the ability of cancer cells to take advantage of normal homeostatic events, such as epithelial-to-mesenchymal transition, or existing cellular machinery to help promote cancer cell survival and progression. In normal tissues, activation of the purinergic pathway and, subsequently, barrier function, by hypoxia, inflammation, ischemia, or acute tissue damage represents a physiological response to maintain cell and tissue homeostasis. In diseases, including the chronic lung diseases asthma, chronic obstructive pulmonary disease, and interstitial lung disease (reviewed, (235)); the neurodegenerative diseases Huntington's disease (236, 237) and Parkinson's disease (238); the inflammatory bowel diseases ulcerative colitis and Crohn's disease; the cardiovascular diseases, congestive heart failure and cardiomyopathy; and sickle cell disease (239) and renal disease, the normally pro-homeostasis function of this pathway is significantly altered to promote disease pathophysiologies. The purinergic pathway has not been studied in great detail in cancer. Therefore, it is unclear if the purinergic pathway members and extracellular adenosine serve to promote the progression of EC or whether the homeostatic mechanisms of this pathway remain intact in well-differentiated, non-invasive, and non-metastatic ECs. Hypoxia and inflammation have prominent roles in many non-cancer diseases, including those indicated above. Interestingly, hypoxia and inflammation have also been shown in cancers to favor cancer progression, metastasis, and/or therapy resistance (reviewed (240, 241)). These observations led us to hypothesize that the most central members of

the purinergic pathway involved in the generation and regulation of extracellular adenosine would likely be dysregulated in EC.

To accomplish this specific aim, we worked in collaboration with the University of Texas at Houston Quantitative Genomic Core to design probe-based real-time quantitative assays for high-throughput analysis of selected purinergic pathway members. 14 samples of normal endometrium and 49 ECs of varying histotypes were received from the University of Texas MD Anderson Multidisciplinary Gynecological Cancer Translational Research Tumor Bank (MDA-GCTR-TB). Table 5.1 indicates the pathological and clinical characteristics of the ECs. Selected purinergic pathway members assessed included AMP → adenosine catalyzing ecto-enzyme 5' nucleotidase (*CD73*); adenosine → inosine deaminating enzyme, adenosine deaminase (*ADA*); nucleoside transporter, equilibrative nucleoside transporter 1 (*ENT1*); adenosine receptor subtypes, *A1R*, *A2AR*, *A2BR*, and *A3R*; P2 receptor, purinergic receptor P2Y (*P2RY2*); and osteopontin (*SPP1*). *SPP1* is not a member of the purinergic pathway, but it has been described as an indicator of elevated extracellular adenosine levels in models of chronic lung diseases (242, 243).

Table 5.1. Pathological and clinical characteristics of endometrial carcinomas

	G1 EEC (n=11)	G2 EEC (n=17)	G3 EEC (n=17)	UPSC (n=4)
Myometrial Invasion, n(%)				
Non-Invasive	3 (27%)	2 (12%)	0 (0%)	0 (0%)
<50%	5 (45%)	10 (59%)	2 (12%)	2 (50%)
= or >50%	3 (27%)	5 (29%)	15 (88%)	2 (50%)
FIGO Stage, n(%)				
IA	3 (27%)	2 (12%)	0 (0%)	0 (0%)
IB	5 (45%)	7 (41%)	2 (12%)	1 (25%)
IC	2 (18%)	2 (12%)	4 (23%)	0
II	0 (0%)	4 (23%)	4 (23%)	0
III	1 (9%)	2 (12%)	4 (23%)	1 (25%)
IV	0 (0%)	0 (0%)	3 (18%)	2 (50%)
Vascular/Lymphatic Invasion, n(%)				
Positive	2 (18%)	5 (29%)	12 (71%)	3 (75%)

Abbreviations:

G1 EEC, Grade 1 Endometrioid Endometrial Carcinoma

G2 EEC, Grade 2 Endometrioid Endometrial Carcinoma

G3 EEC, Grade 3 Endometrioid Endometrial Carcinoma

UPSC, Uterine Papillary Serous Carcinoma

FIGO, International Federation of Gynecology and Obstetrics

FIGO Stage:

IA, cancer confined to endometrial layer

IB, cancer confined to uterine corpus, but invades less than half the myometrium thickness

IC, cancer confined to uterine corpus, but invades equal to or more than half the myometrium thickness

II, cancer spreads to the cervix, but does not extend beyond the uterus

III, local and/or regional spread of the cancer

IV, cancer has spread to distant tissues or organs

Ecto-enzyme 5' nucleotidase (*CD73*) is the single purinergic pathway member dysregulated in endometrial carcinomas

Results from high-throughput qRT-PCR analyses were assessed by comparing transcript levels to established pathological and surgical parameters for EC, including histological grade, myometrial invasion, surgical-pathological staging (FIGO), disease type (Type I vs. Type II), and vascular/lymphatic invasion. This information was derived from review of the pathology reports for each EC examined. Type I includes Grade 1 and Grade 2 endometrioid endometrial carcinomas (G1 EEC and G2 EEC), and Type II included Grade 3 endometrioid endometrial carcinomas (G3 EEC) and uterine papillary serous carcinomas (UPSC). Ecto-enzyme 5' nucleotidase (*CD73*) was found to be the single purinergic pathway member to be consistently and significantly dysregulated in normal versus EC and among ECs and their different parameters (Table 5.2 and 5.3). Therefore, for subsequent comparisons to pathological and clinical data (Figures 5.2-5.5), only *CD73* was considered. Figure 5.1 indicates expression of selected members of the purinergic pathway in proliferative and secretory endometrium and by endometrial carcinoma grade.

Table 5.2. One-Way ANOVA and independent t-test *p* values for purinergic pathway members evaluated by qRT-PCR in normal endometrium and endometrial carcinomas – Part I

	Gene Symbol	Grade <i>a p</i>	Myometrial Invasion <i>a p</i>	FIGO Stage <i>a p</i>
5'Nucleotidase	<i>CD73</i>	7.0x10 ⁻²⁰	0.019	0.037
Adenosine Deaminase	<i>ADA</i>	0.733	0.760	0.258
Adenosine Receptor A1	<i>A1R</i>	0.798	0.672	0.111
Adenosine Receptor A2A	<i>A2AR</i>	0.886	0.694	0.468
Adenosine Receptor A2B	<i>A2BR</i>	0.879	0.969	0.099
Adenosine Receptor A3	<i>A3R</i>	0.264	0.253	0.267
Equilibrative Nucleoside Transporter 1	<i>ENT1</i>	0.036	0.043	0.056
Osteopontin	<i>SPP1</i>	0.013	0.324	0.911
Purinergic Receptor P2Y	<i>P2RY2</i>	0.442	0.906	0.128

^aOne-Way ANOVA statistics were used, significance was determined by a *p* value of *p*<0.05

Abbreviations:

FIGO, International Federation of Gynecology and Obstetrics

Table 5.3. One-Way ANOVA and independent t-test *p* values for purinergic pathway members evaluated by qRT-PCR in normal endometrium and endometrial carcinomas – Part II

	Gene Symbol	Type I vs Type II <i>bp</i>	Vascular/Lymphatic Invasion <i>bp</i>
5'Nucleotidase	<i>CD73</i>	0.001	0.616
Adenosine Deaminase	<i>ADA</i>	0.288	0.146
Adenosine Receptor A1	<i>A1R</i>	0.466	0.536
Adenosine Receptor A2A	<i>A2AR</i>	0.122	0.933
Adenosine Receptor A2B	<i>A2BR</i>	0.466	0.083
Adenosine Receptor A3	<i>A3R</i>	1.000	0.664
Equilibrative Nucleoside Transporter 1	<i>ENT1</i>	0.125	0.072
Osteopontin	<i>SPP1</i>	0.052	0.302
Purinergic Receptor P2Y	<i>P2RY2</i>	0.177	0.312

^bIndependent t-test statistics were used, significance was determined by a *p* value of $p < 0.05$

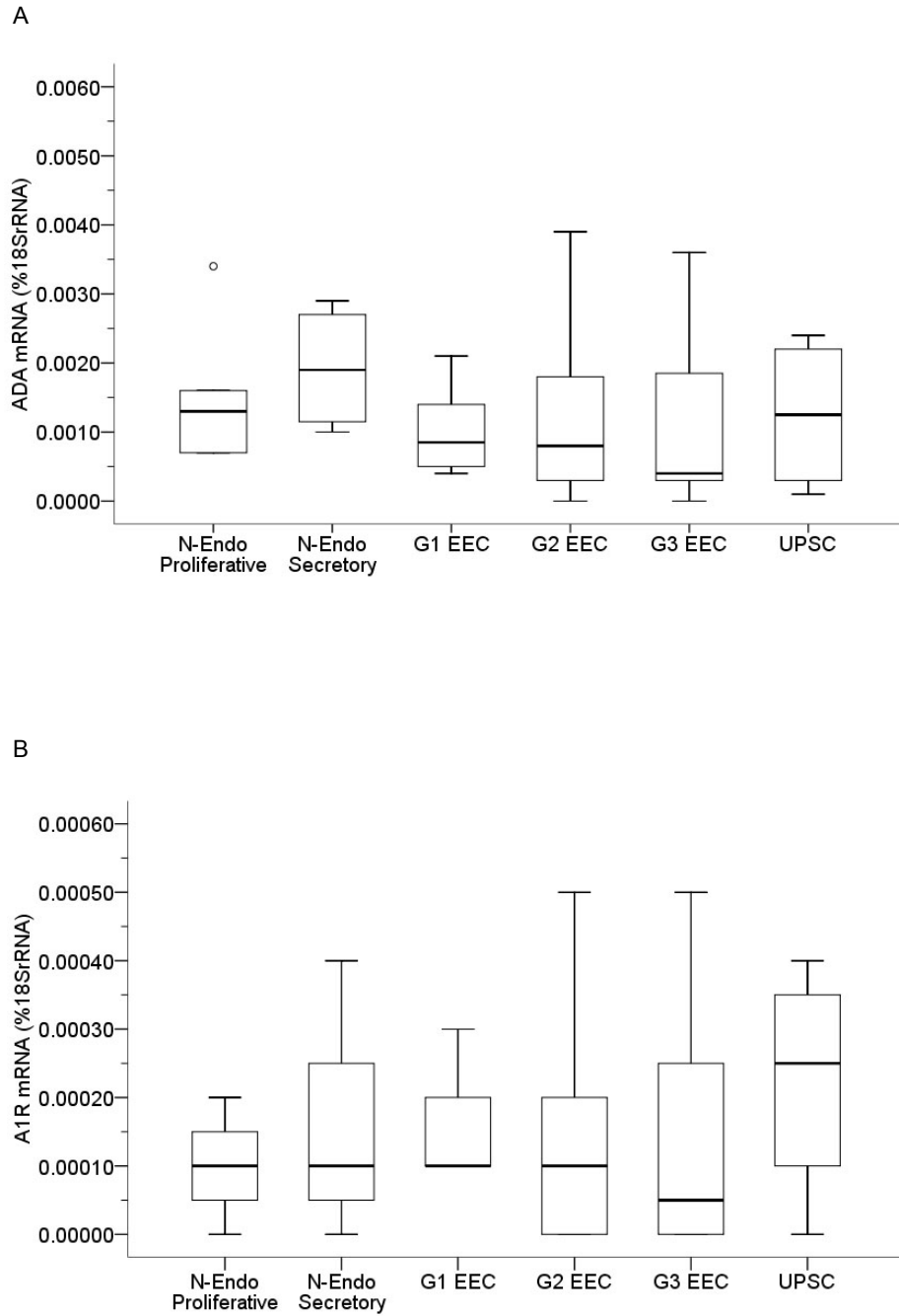
Categories:

Type I, Grade 1 and Grade 2 endometrioid endometrial carcinoma

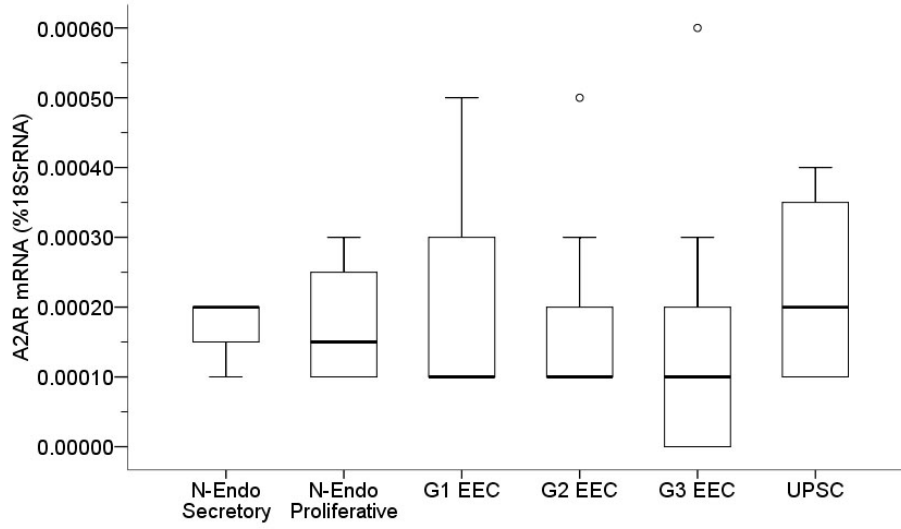
Type II, Grade 3 endometrioid endometrial carcinoma and uterine papillary serous carcinoma

Figure 5.1. Expression of selected members of the purinergic pathway in proliferative and secretory endometrium and by endometrial carcinoma grade. Transcript data were plotted using a box and whisker plots: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent; circles, outside fence; and asterisks, extreme values. *CD73* transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%18SrRNA). (A) *ADA* transcripts, (B) *AIR* transcripts, (C) *A2AR* transcripts, (D) *A2BR* transcripts, (E) *A3R* transcripts, (F) *ENT1* transcripts, (G) *SPP1* transcripts, and (H) *P2RY2* transcripts. Error bars represent ± 1 SE. (ns = non-significant; *, $p < 0.05$)

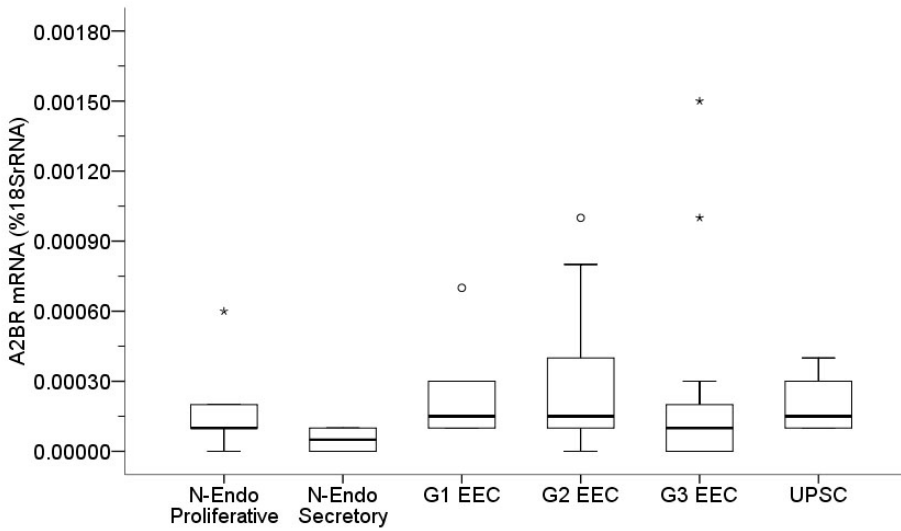
Figure 5.1



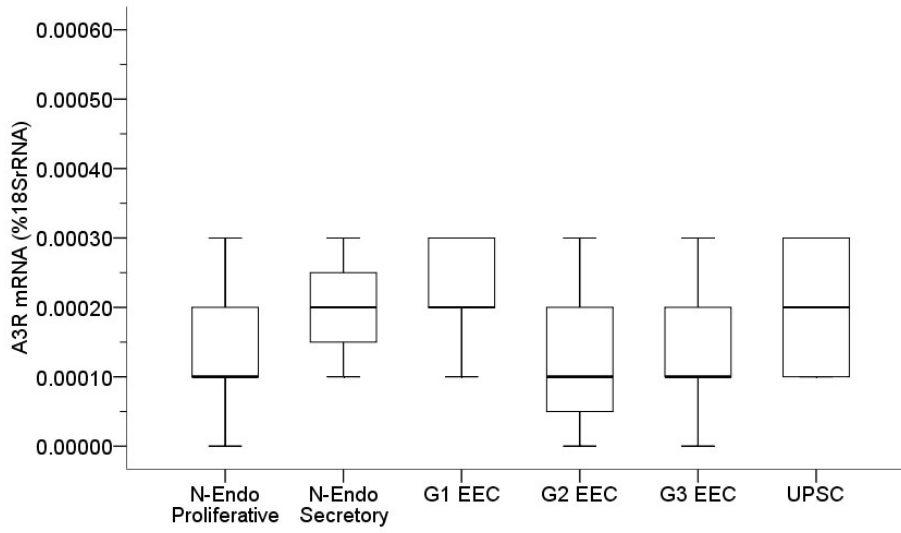
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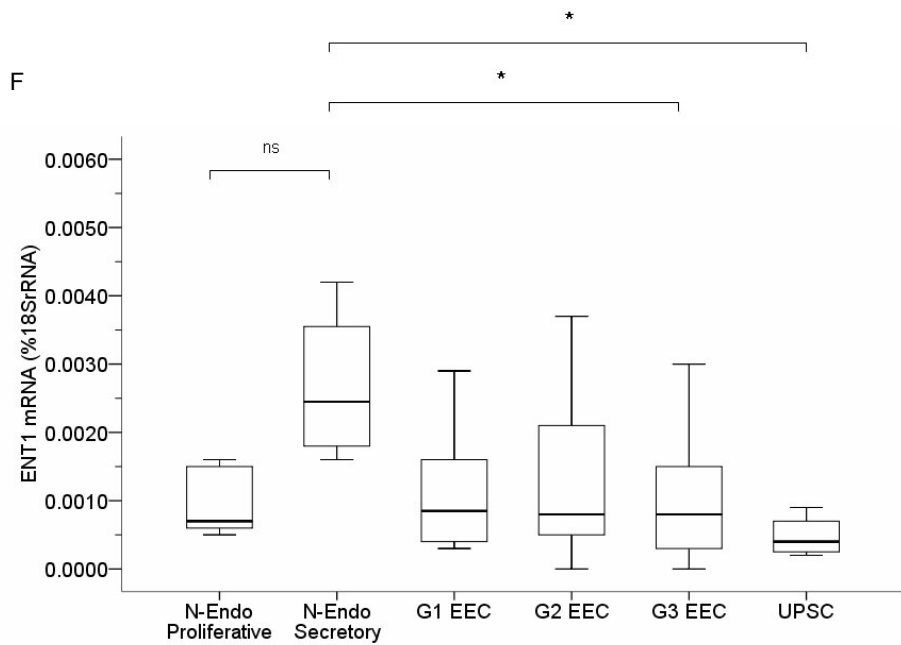
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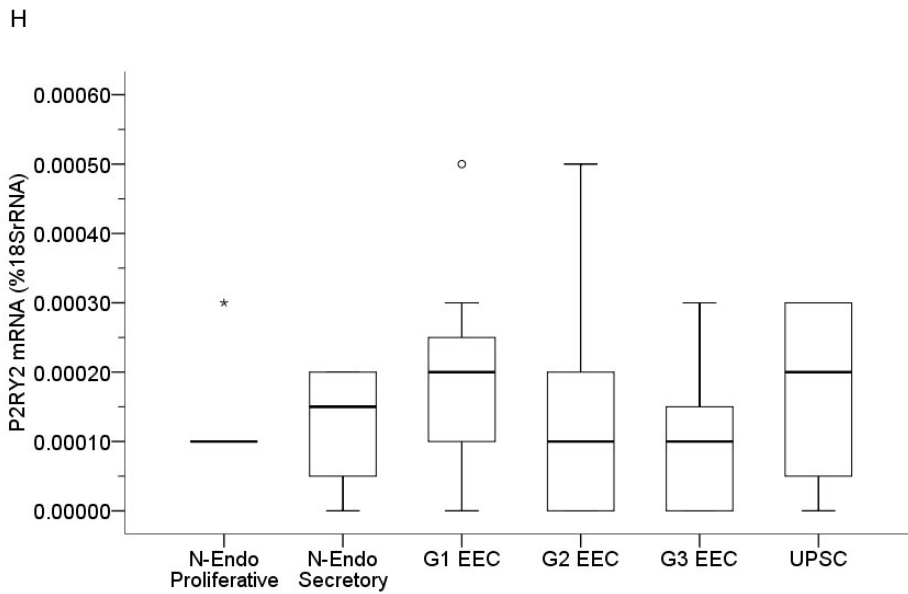
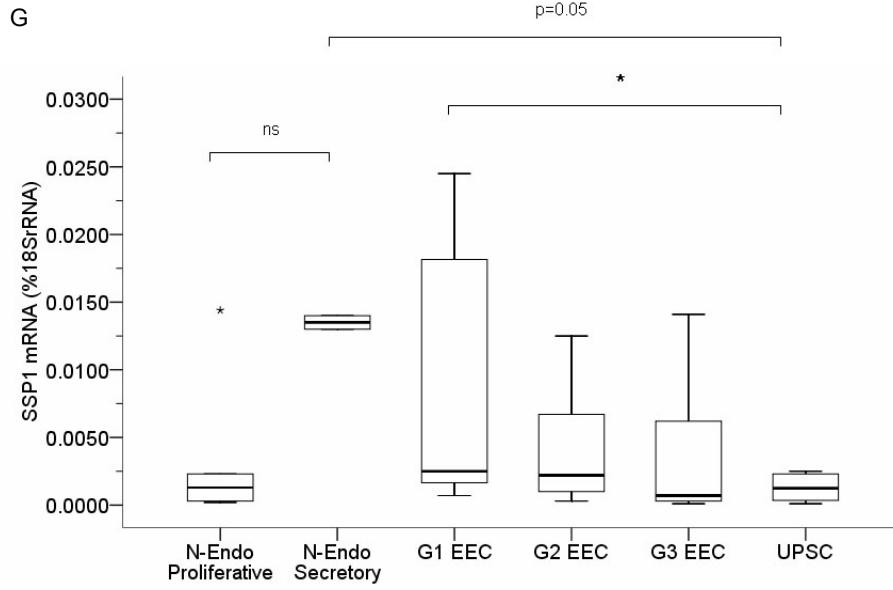


E



F





Elevated *CD73* expression in secretory phase endometrium is not regulated by progesterone.

A unique feature of the normal endometrium is its cyclic regulation by estrogen and progesterone. Proliferative phase endometrium is dominated by changes induced by estrogen, while secretory phase endometrium is dominated by changes induced by progesterone (244). Prior to performing post-hoc analyses for *CD73* transcripts in EC, *CD73* expression was assessed among the two phases of normal endometrium. Interestingly, in normal secretory endometrium *CD73* transcripts were significantly elevated as compared to normal proliferative endometrium (Figure 5.2). This finding was exciting, as epithelial cells of secretory phase endometrium are the most differentiated of the two phases and are highly supportive of barrier function and fertility. With progesterone being the most dominant hormone of the secretory phase and it being responsible for the regulation of many genes in this phase, the possibility of progesterone inducing *CD73* expression was assessed by using well-differentiated endometrial carcinoma cells, HEC-1A, manipulated to stably express progesterone receptor β (PR β) (245). Somewhat unexpected, progesterone was found not to induce *CD73* transcripts (Figure 5.3 A). In a similar regard, *A2BR* transcripts were not induced by progesterone (Figure 5.3 B). Insulin-like growth factor-binding protein 1 (*IGFBP1*), a known progesterone regulated gene, was induced in the same experimental conditions (Figure 5.3 C). *CD73* and *A2BR* induction by progesterone was additionally assessed in endometrial biopsies from women taking the progestin, depot medroxyprogesterone acetate (MPA). Patients who took depot MPA were premenopausal and biopsies were timed at days 5-10 in the proliferative phase. Biopsies were taken at baseline (prior to treatment) and 3 months post-treatment. *CD73* transcripts were not increased in endometrial biopsies. In most patients, in fact, *CD73* expression was down-regulated (Figure 5.4 A and Table 5.4). *A2BR* transcripts remained unchanged or were elevated in a small number of patients at 3 months post-treatment (Figure 5.4 B and Table 5.4). These studies demonstrate *CD73* transcripts to be significantly elevated in secretory phase endometrium, a phase known to be characterized by enhanced epithelial

differentiation and barrier function. However, expression of *CD73* and *A2BR* are not regulated by the dominant hormone of the secretory phase, progesterone.

Figure 5.2. *CD73* expression in proliferative and secretory phase endometrium. *CD73* transcript data from qRT-PCR studies of proliferative and secretory phase endometrium were plotted using a box and whisker plot: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent; circles, outside fence. *CD73* transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%18SrRNA). *CD73* transcripts were significantly elevated in secretory phase endometrium. Error bars represent ± 1 SE. (** $p < 0.005$)

Figure 5.2

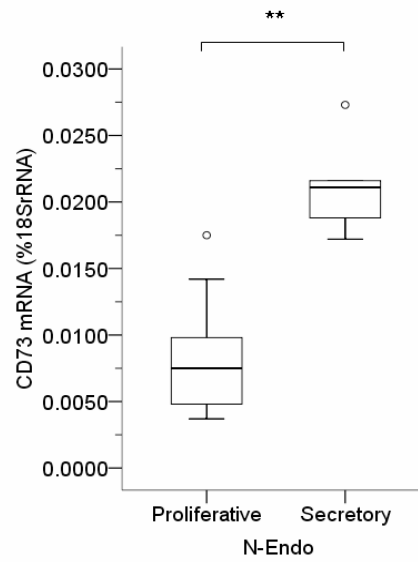


Figure 5.3. Progesterone treatment in endometrial carcinoma cells, HEC-1A, manipulated to express progesterone receptor β (PR β). HEC-1A stably expressing progesterone receptor β (PR β) were treated with 400nM of progesterone or vehicle control (0.001%, 100% ethanol (EtOH)) for 24 hours and transcripts for *CD73*, *A2BR*, and insulin-like growth factor-binding protein 1 (*IGFBP1*) assessed by qRT-PCR. *CD73* (A) and *A2BR* (B) transcripts were not induced by progesterone. (C) Known progesterone regulated gene and positive control *IGFBP1* was induced by progesterone. Transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of transcript/molecules of *18S* rRNA (%18SrRNA). Error bars represent \pm 1 SE. (***) $p < 0.0005$)

Figure 5.3

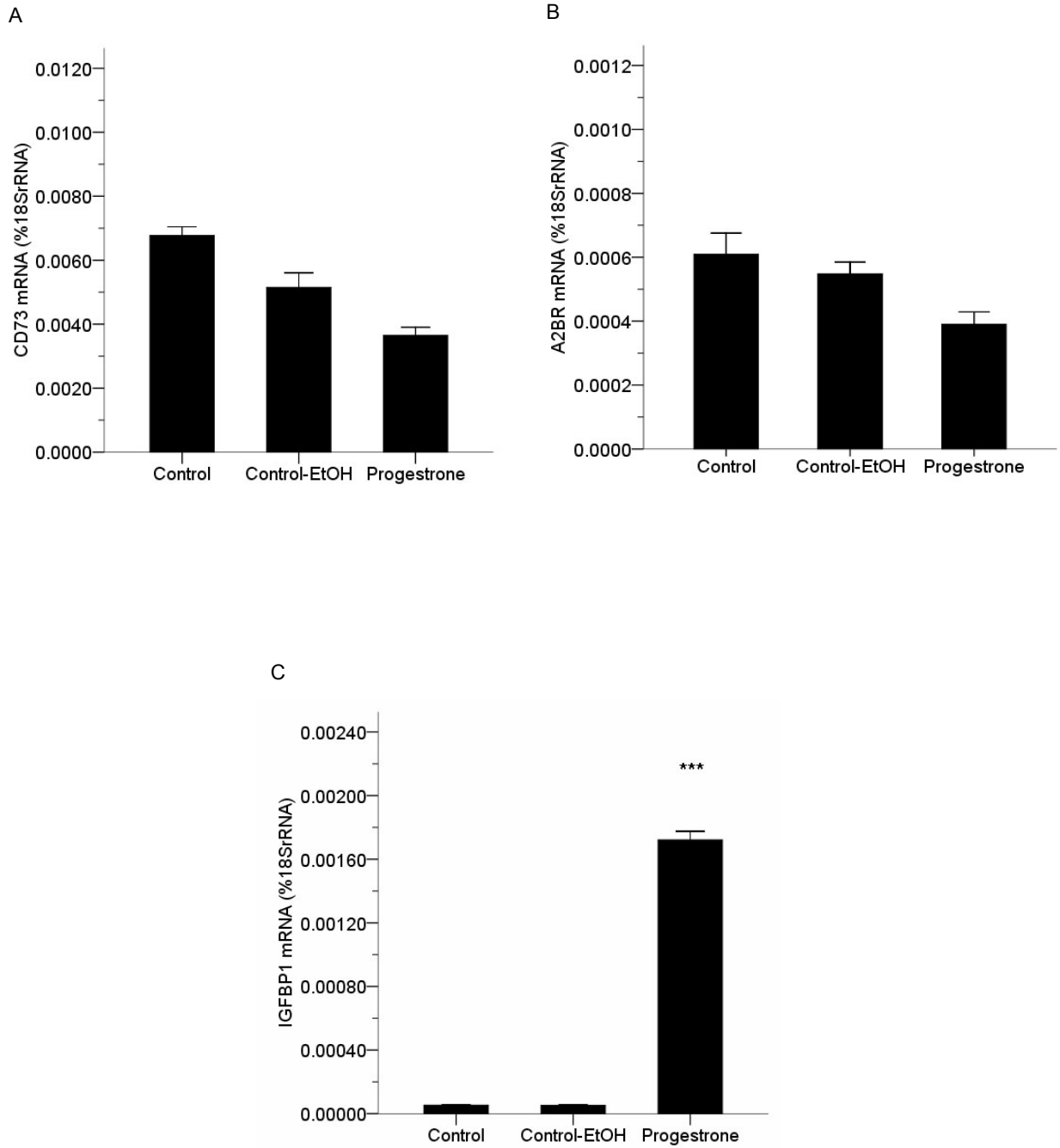


Figure 5.4. *CD73* and *A2BR* expression in benign endometrium at baseline and 3 months post-progestin, depot medroxyprogesterone acetate (MPA), treatment. Baseline and 3 month post-treatment endometrial biopsies were obtained from women taking exogenous progesterone, depot medroxyprogesterone acetate (MPA). *CD73* and *A2BR* transcripts were assessed by qRT-PCR. Transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%18SrRNA). Individual lines represent a single patient. (A) *CD73* transcripts remained unchanged or were down-regulated in patients following 3 months post-MPA as compared to baseline. (B) *A2BR* transcripts remained unchanged or were increased in patients following 3 months post-MPA as compared to baseline.

Figure 5.4

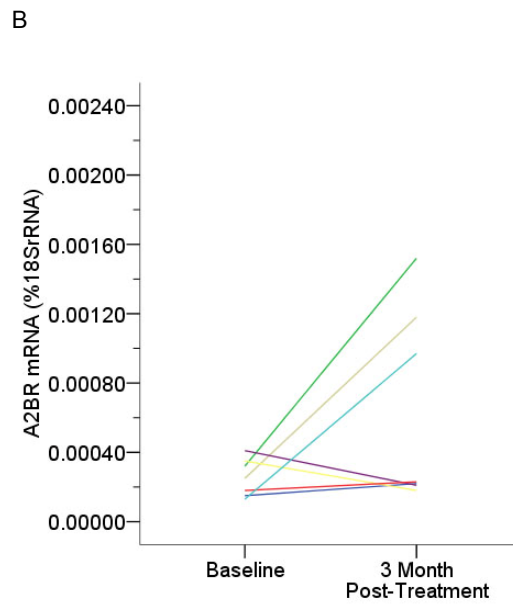
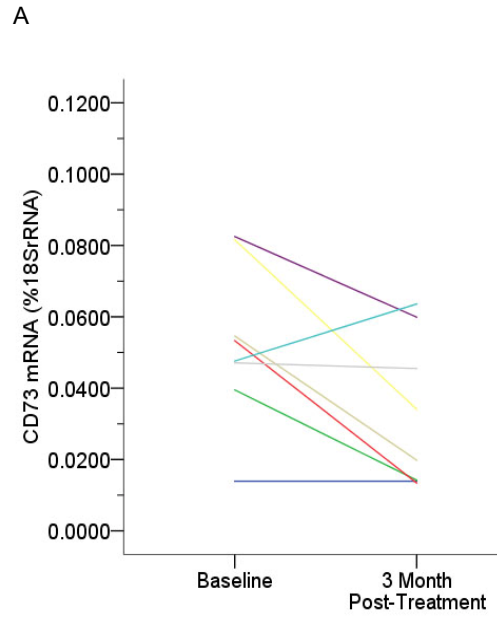


Table 5.4. *CD73* and *A2BR* expression in benign endometrium at baseline and 3 months post-progestin, depot medroxyprogesterone acetate (MPA), treatment – Data summary from Figure 5.4

Patient	Line	CD73 Baseline	CD73 3 Month Post-Treatment
1	Purple	0.0825	0.0599
2	Yellow	0.0816	0.0341
3	Teal	0.0546	0.0198
4	Gray	0.0533	0.0134
5	Tan	0.0476	0.0636
6	Red	0.0471	0.0455
7	Green	0.0395	0.0142
8	Blue	0.0139	0.0139

Patient	Line	A2BR Baseline	A2BR 3 Month Post-Treatment
1	Green	0.00032	0.00152
2	Tan	0.00025	0.00118
3	Teal	0.00013	0.00097
4	Purple	0.00041	0.00021
5	Yellow	0.00035	0.00018
6	Red	0.00018	0.00023
7	Blue	0.00015	0.00022

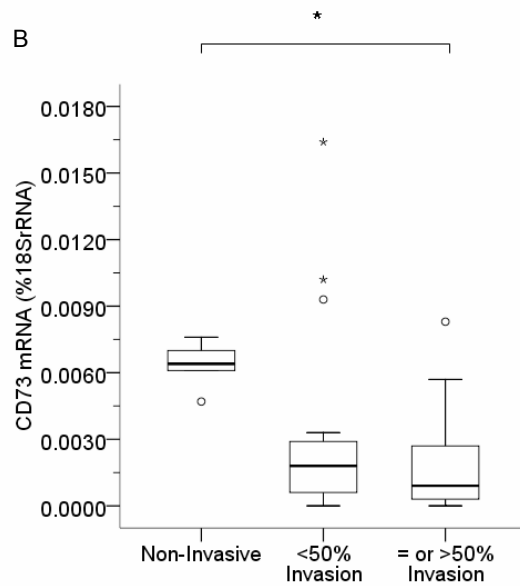
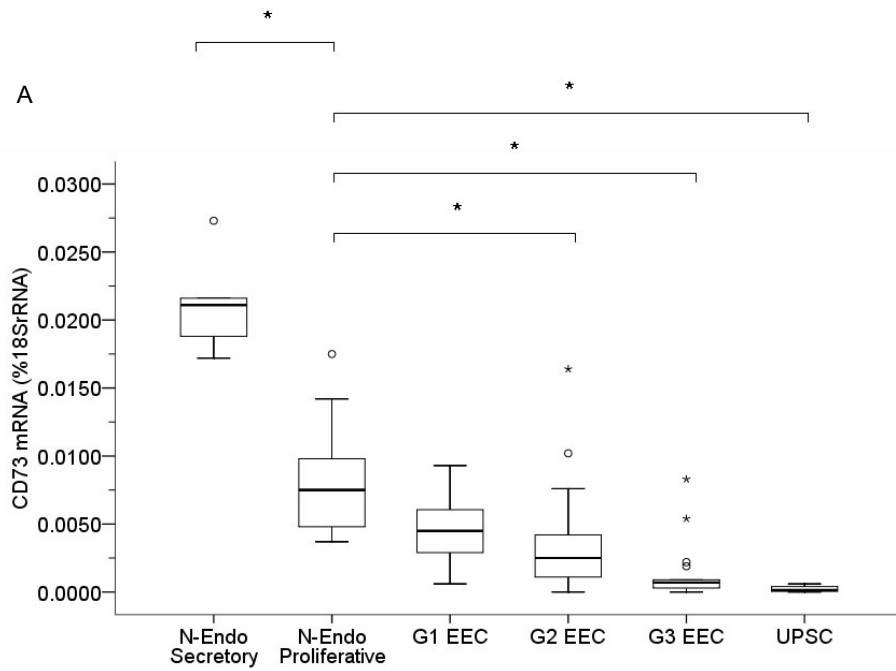
Data are expressed as molecules of *CD73*/molecules of *18S* ribosomal RNA for a single patient

***CD73* expression is down-regulated in moderately- and poorly-differentiated, invasive, and metastatic endometrial carcinomas**

Post-hoc analyses (Tukey) was performed to determine *CD73* transcript expression in relation to EC histotype and grade, invasiveness, and stage. G1 EECs are well-differentiated, G2 EECs are moderately-differentiated, and G3 EEC and UPSC are poorly-differentiated. Compared to expression in secretory phase endometrium, *CD73* transcripts were significantly lower in all the different grades of EC (Figure 5.5 A). Proliferative endometrium and G1 EECs had similar levels of *CD73* transcripts, but compared to proliferative endometrium, *CD73* expression in G2 and G3 EECs and UPSCs was significantly lower. To assess differences in myometrial invasion, ECs were divided into three categories: non-invasive, less than (<) 50% invasion, and equal to (=) or greater than (>) 50% invasion (Figure 5.5 B). Non-invasive are ECs confined completely to the endometrium. ECs with 50% or greater myometrial invasion had significantly lower *CD73*. In a similar manner, ECs were divided into surgical staging categories (Figure 5.5 C). FIGO stage IA ECs are non-invasive, FIGO stage IB & IC ECs invade the myometrium but are confined to the uterine corpus, and FIGO II, III & IV ECs have extrauterine disease involvement, whether local (II), regional (III), or distant (IV). *CD73* transcripts were lower ($p=0.05$) for FIGO stage IB & IC ECs compared to the non-invasive stage IA ECs. *CD73* was significantly lower in stage II, III, & IV ECs compared to the non-invasive stage IA ECs. When ECs are grouped into Type I or Type II categories, *CD73* transcripts were found to be significantly lower in Type II disease (Figure 5.5 D). As previously known from our One-Way ANOVA statistical comparisons, vascular/lymphatic invasion was not a significant determinant of *CD73* expression (Figure 5.5 E). These data indicate that at the transcript level, *CD73* is significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs. *CD73* transcripts levels in non-invasive were similar to those seen with benign proliferative phase endometrium.

Figure 5.5. *CD73* expression in established pathological and clinical parameters for endometrial cancer. *CD73* transcript data were plotted using a box and whisker plots: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent; circles, outside fence; and asterisks, extreme values. *CD73* transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%*18SrRNA*). (A) *CD73* transcripts in different grades of EC were significantly different among all groups when compared with secretory phase endometrium. *CD73* transcripts of proliferative and Grade 1 endometrioid endometrial carcinomas (G1 EEC) were similar. *CD73* in Grade 2 and Grade 3 endometrioid endometrial carcinomas (G2 EEC and G3 EEC) and uterine papillary serous carcinomas (UPSC) were significantly lower. (B) *CD73* transcripts were significantly lower in invasive ECs with 50% or more myometrial invasion compared to non-invasive ECs. (C) Compared to non-invasive stage IA ECs, *CD73* transcripts were at statistical significance for stage IB and IC ECs ($p=0.05$) and significantly lower in more advanced stage ECs ($p<0.05$). (E) *CD73* transcripts were significantly lower in Type II ECs ($p<0.05$). (F) Vascular/lymphatic invasion was not a significant determinant of *CD73* expression. Error bars represent ± 1 SE.

Figure 5.5



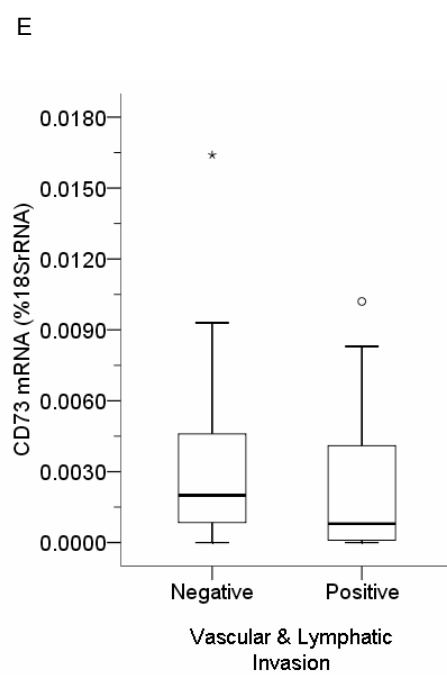
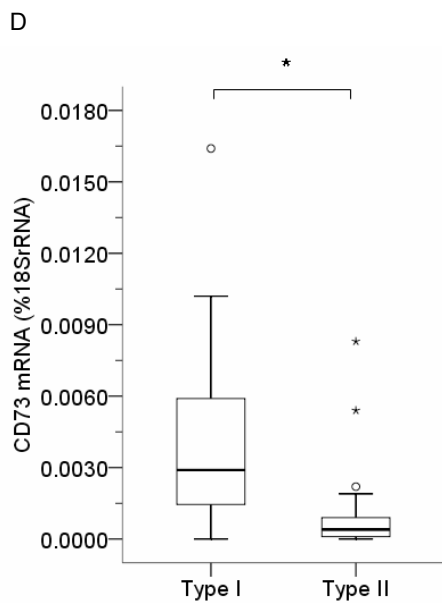
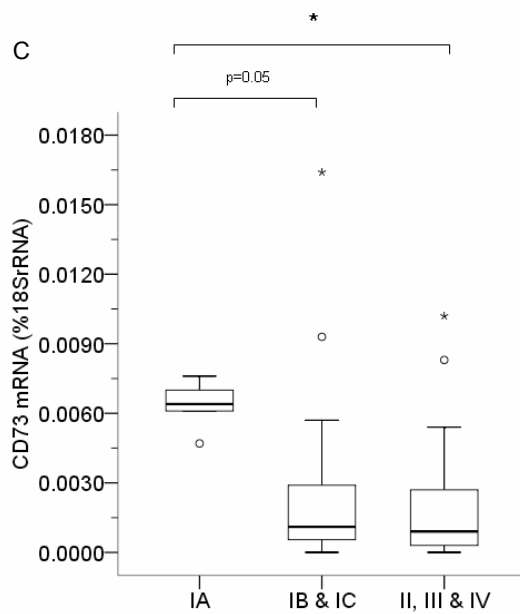


Table 5.5. CD73 expression in established pathological and surgical parameters for EC –

Data summary from Figure 5.5

	N	Mean ± SE	
Normal Endometrium			
N-Endo Proliferative	9	0.0087 ± 0.0015	a
N-Endo Secretory	5	0.0212 ± 0.0017	
Grade			
N-Endo Secretory	5	0.0212 ± 0.0017	
N-Endo Proliferative	9	0.0087 ± 0.0015	a
G1 EEC	11	0.0046 ± 0.0070	a
G2 EEC	17	0.0037 ± 0.0010	a,b
G3 EEC	17	0.0014 ± 0.0005	a,b
UPSC	4	0.0002 ± 0.0001	a,b
Myometrial Invasion			
Non-Invasive	5	0.0064 ± 0.0050	
<50% Invasion	19	0.0031 ± 0.0010	
= or >50% Invasion	25	0.0019 ± 0.0004	c
FIGO			
IA	5	0.0064 ± 0.0005	
IB & IC	23	0.0026 ± 0.0008	
II, III, IV	21	0.0022 ± 0.0006	d
Type I vs Type II			
Type I	28	0.0041 ± 0.0007	
Type II	21	0.0012 ± 0.0004	e
Vascular & Lymphatic Invasion			
Negative	27	0.0033 ± 0.0007	
Positive	22	0.0023 ± 0.0006	

Data are expressed as mean ± SE molecules of CD73/molecules of 18S ribosomal RNA

aIndicates significant difference (p<0.05) compared with N-Endo Secretory

bIndicates significant difference (p<0.05) compared with N-Endo Proliferative

cIndicates significant difference (p<0.05) compared with Non-Invasive

^dIndicates significant difference ($p<0.05$) compared with FIGO IA

^eIndicates significant difference ($p<0.05$) compared with Type I

Abbreviations and Categories:

G1 EEC, Grade 1 Endometrioid Endometrial Carcinoma

G2 EEC, Grade 2 Endometrioid Endometrial Carcinoma

G3 EEC, Grade 3 Endometrioid Endometrial Carcinoma

UPSC, Uterine Papillary Serous Carcinoma

FIGO, International Federation of Gynecology and Obstetrics

Type I, Grade 1 and Grade 2 endometrioid endometrial carcinoma

Type II, Grade 3 endometrioid endometrial carcinoma and uterine papillary serous carcinoma

Down-regulation of *CD73* is not limited to endometrial cancer

From Figure 5.5 A and Table 5.5, it was observed that uterine papillary serous carcinomas (UPSC) have very low *CD73*. This EC histotype is typically very aggressive, associated with metastatic disease at time of diagnosis, and poor clinical outcome. High grade papillary serous carcinoma is also a clinically aggressive variant of ovarian carcinoma. Microscopically, ovarian high grade papillary serous carcinoma (HGpsc) is very similar to uterine papillary serous carcinoma (UPSC). Therefore, *CD73* was also examined in this subtype of ovarian cancer. In Figure 5.6, HGpsc's have significantly lower *CD73*, similar to the low values seen in UPSC. This data highlights that *CD73*'s importance is not limited to EC.

Figure 5.6. *CD73* expression in normal ovary and ovarian high grade papillary serous carcinoma (HGPSC). *CD73* transcript data from qRT-PCR studies of normal ovary and high grade papillary serous carcinomas (HGPSC) were plotted using a box and whisker plot: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent; circles, outside fence. *CD73* transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%18SrRNA). *CD73* transcripts were significantly down-regulated in HGPSC. Error bars represent ± 1 SE. (* $p < 0.05$)

Figure 5.6

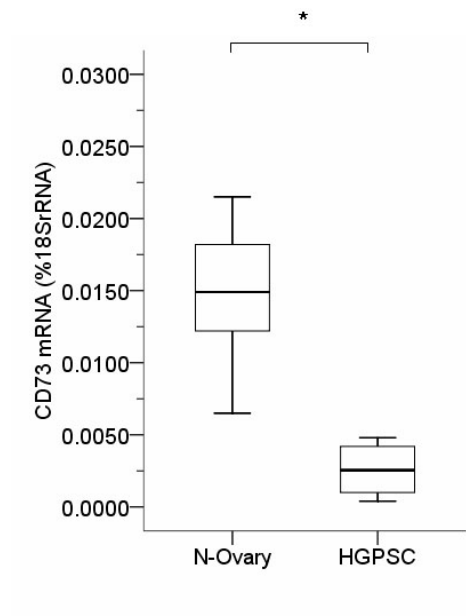


Table 5.6. *CD73* expression in normal ovary and ovarian high grade papillary serous carcinoma (HGpsc) – Data summary from Figure 5.6

	N	Mean ± SE	p value (N-Ovary vs HGpsc)
N-Ovary	9	0.0146 ± 0.0017	--
HGpsc	10	0.0027 ± 0.0005	0.00006

Data are expressed as mean ± SE molecules of *CD73*/molecules of *18S* ribosomal RNA

Summary

Generation of extracellular adenosine is important in maintaining homeostasis in normal epithelial cells. However, this pro-homeostasis function of the purinergic pathway is often times significantly altered to promote disease pathophysiologies. The purinergic pathway has not been studied in great detail in cancer. qRT-PCR studies of select members of the purinergic pathway identified CD73, the primary ecto-enzyme responsible for the generation of extracellular adenosine, to be the single member significantly dysregulated in EC. CD73 was found to be significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic EC as compared to normal endometrium, proliferative or secretory, or well-differentiated and non-invasive ECs. CD73 is the primary ecto-enzyme responsible for the generation of extracellular adenosine. These data suggest that the down-regulation of the homeostatic action of extracellular adenosine may be important for the progression of this disease. EC is a cancer that arises in the epithelial cells of the endometrium. The most related pro-homeostasis event of CD73-generated adenosine is to induce barrier function in endothelial cells and intestinal epithelial cells. Barrier function is a homeostatic event whose actions are directly related to the regulation and maintained integrity of cell-cell adhesions and their individual proteins. The loss of cell-cell adhesions in carcinoma cells significantly influences carcinoma cell progression and metastasis. The following specific aims and their studies address the potential relationship of CD73-generated adenosine is to induce the homeostatic event of barrier function in epithelial cells of the normal endometrium and the relatedness of this homeostatic action to inhibit the progression of EC cells, potentially by regulating cell-cell adhesions.

CHAPTER SIX

Results

Specific Aim 2. Demonstrate that CD73-generated adenosine serves a critical barrier protective role in epithelial cells of the endometrium and that CD73 expression is induced by known stimuli of barrier function.

Introduction

The barrier function induced by extracellular adenosine is predominantly a response related to cellular stress. This is best demonstrated by the understanding that mice deficient for CD73 (205) or A2BR (206) have unremarkable phenotypes at conditions equal to basal physiology. However, both of these transgenic mice suffer extensive vascular leak and pulmonary edema when placed in hypoxic conditions (205, 206). Increased production of extracellular adenosine and its effect on barrier protection is related to the concomitant increase of CD73 and A2BR by stressors. This increase is related to events such as cytokine release (207), or hypoxia-induced HIF-1 transcriptional activity (204, 210). Studies in specific aim 1 suggest that CD73's function in normal endometrium is counterproductive for EC progression, as *CD73* transcripts were found to be significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs as compared to normal endometrium or well-differentiated and/or non-invasive ECs.

Specific aim 2 examines two of the central themes of adenosine's barrier function: 1.) adenosine's barrier function is a product of CD73's generation of adenosine and its activation of the adenosine receptor subtype, A2BR; and 2.) adenosine's barrier function effect is supported by changes in the expression of purinergic pathway members to support the extracellular accumulation of adenosine. Sub-aim 2A was intended to establish that CD73-generated adenosine and A2BR are important to endometrial epithelial barrier function *in vivo*. To accomplish this

sub-aim, an *in vivo* uterine perfusion assay was developed that takes advantage of the electron dense properties of ruthenium red. Ruthenium red is a low molecular weight dye, 786 daltons, that binds to cell surface acid polysaccharides (246). With loss of barrier function, ruthenium red will move into paracellular spaces (229), and this can be visualized using transmission electron microscopy. This is an established method for measuring the integrity of barrier function of epithelial cells (229). Mice deficient for CD73 ($CD73^{-/-}$) or A2BR ($A2BR^{-/-}$) were used in these studies alongside with wild-type (C57BL/6) mice. For sub-aim 2B, the ability of the cytokine transforming growth factor- β 1 (TGF- β 1) or increased steady-state levels of HIF-1 α to increase CD73 and/or A2BR expression in EC cells was assessed. Sub-aim 2B was intended to investigate whether EC cells retain the capability to induce CD73 and/or A2BR when stressed and thus provide the chance to induce barrier function by adenosine. TGF- β 1 was used as a way to mimic the release of cytokines on epithelial cells, as seen in inflammation, and hypoxia was used to increase the steady-state levels of HIF-1 α . HIF-1 is a heterodimer consisting of HIF-1 α and HIF-1 β subunits (247, 248). The biological activity of HIF-1 is determined by the expression and activity of the HIF-1 α subunit (249-251). In response to regain O₂ homeostasis in stressed cells, HIF-1 α steady-state levels increase and leads to HIF-1 driven-gene expression (reviewed, (252)). HIF-1 binding sequences in CD73 and A2BR promoters have been described, and HIF-1 directly induces CD73 (204) and A2BR (210) expression. TGF- β 1 has been established as an inducer of intestinal epithelial barrier function (188-193). However, TGF- β 1's-mediated barrier function has not been previously linked to CD73, adenosine, or A2BR expression or activity. This specific aim is the first to report such findings.

***In vivo* CD73 or A2BR deficiency causes loss of barrier function in endometrial epithelial cells**

The results obtained in specific aim 1 suggest that the loss of CD73 function in normal endometrium and well-differentiated ECs is important for the progression of this disease. Generation of adenosine at the extracellular surface in normal cell types and tissues is an important homeostatic response in response to cellular stress. Adenosine's extracellular activity induces angiogenesis, vasodilatation, immunosuppression, ischemic pre-conditioning and post-conditioning, balancing the oxygen supply/demand ratio, inducing endothelial and epithelial barrier function, inhibiting superoxide generation, and inhibiting excitatory neurotransmitter release (reviewed, (184)). We considered that CD73-generated adenosine may be an important component for barrier function in the normal endometrial epithelium. To determine if CD73 or A2BR deficiency was associated with the loss of barrier function in endometrial epithelial cells, an *in vivo* ruthenium red paracellular permeability assay that takes advantage of the electron density of ruthenium red was developed. For barrier function stress, 10-14 week old, proestrus phase mice were maintained in hypoxia conditions (7% O₂) for 4 hours. Controls were maintained in normoxia conditions (room air, 21% O₂). Studies have shown that CD73^{-/-} (205) and A2BR^{-/-} (206) mice exposed to hypoxia (8% O₂) have significant endothelial and intestinal epithelial barrier disruption within 4 hours. 7% O₂ was selected for our studies based on our preliminary results using varying hypoxic levels (7%, 8%, or 10% O₂). Uterine horns were perfused-fixed with 0.2% ruthenium red and 2% glutaraldehyde. Ruthenium red paracellular permeability was quantified by assessing approximately 200 consecutive paracellular spaces per uterine section. As shown in Figure 6.1 and Table 6.1, there were no differences in barrier function in normoxia conditioned C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice. In hypoxia, CD73^{-/-} and A2BR^{-/-} mice have significant increases in ruthenium red paracellular permeability, and thus disrupted barrier function, compared to their C57BL/6 counterparts. Representative electron photomicrographs

demonstrating increased ruthenium red paracellular flux in CD73^{-/-} and A2BR^{-/-} mice are shown in Figure 6.2. As expected, significant increases in paracellular flux were observed in both CD73^{-/-} and A2BR^{-/-} mice in the hypoxic conditions compared to normoxia. Depth of ruthenium red paracellular permeability among groups was not significantly different (Table 6.2).

Figure 6.1. *In vivo* ruthenium red paracellular permeability in endometrial epithelial cells of CD73^{-/-}, A2BR^{-/-} and wild-type (C57BL/6) mice. 10-14 week-old, proestrus phase C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice were placed in normoxia (room air, 21% O₂) or hypoxia (7% O₂) conditions for 4 hours. 6-7 mice were studied per group. Uterine horns were perfused-fixed with 0.2% ruthenium red and 2% glutaraldehyde. Ruthenium red paracellular permeability was quantified by assessing approximately 200 consecutive paracellular spaces per uterine section. Percent ruthenium red permeability was determined by the ratio of dye positive to total paracellular spaces assessed. Individual values are plotted, as well as mean \pm 1 SE, for each group. Barrier function was not significantly different among the normoxia C57BL/6, CD73^{-/-}, and A2BR^{-/-} mice. However, following exposure to hypoxia, ruthenium red paracellular flux was significantly increased in CD73^{-/-} and A2BR^{-/-} mice compared to C57BL/6 mice. This protocol was modified and adapted from Molitons, BA., Falk, SA., and Dahl, RH., Ischemia-induced loss of epithelial polarity role of the tight junction, JCI 1989;84:1334-1339 (229) (*p<0.05)

Figure 6.1

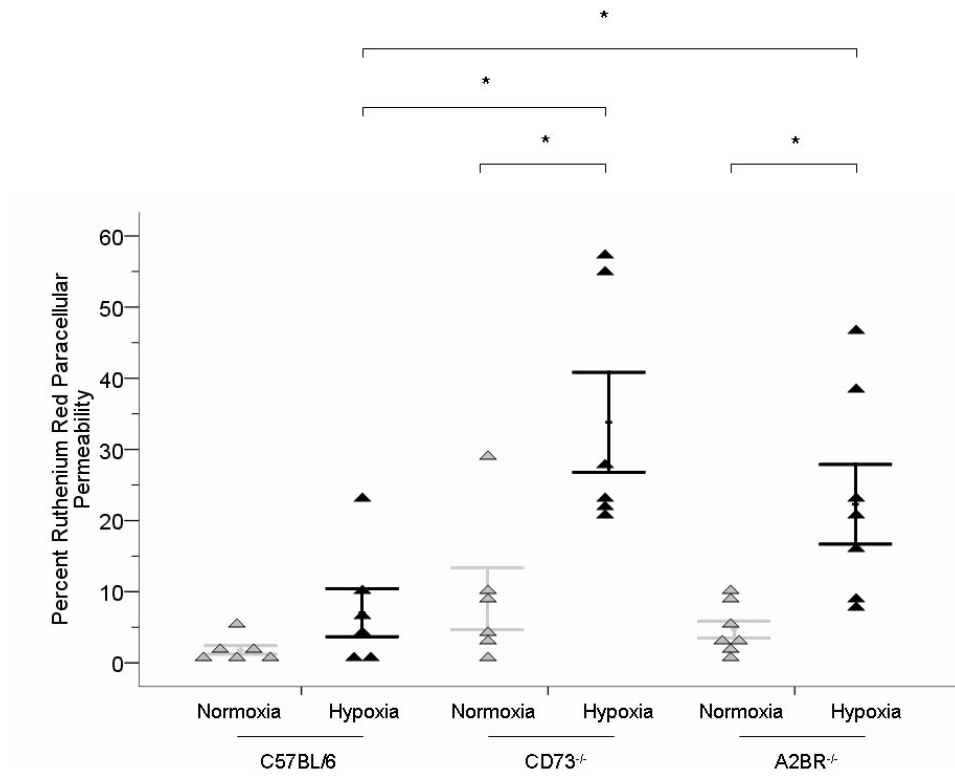


Table 6.1. *In vivo* ruthenium red paracellular permeability in endometrial epithelial cells of CD73^{-/-}, A2BR^{-/-} and wild-type (C57BL/6) mice – Data summary from Figure 6.1

Mouse	N	Mean ± SE (%)	p value (normoxia vs hypoxia)	p value (vs hypoxia C57BL/6)
C57BL/6 normoxia	6	1.82 ± 0.60		
^a C57BL/6 hypoxia	6	7.05 ± 3.36	NS	--
CD73 ^{-/-} normoxia	6	9.01 ± 4.36		
^a CD73 ^{-/-} hypoxia	6	33.82 ± 7.01	0.01	0.01
A2BR ^{-/-} normoxia	7	4.7 ± 1.19		
^a A2BR ^{-/-} hypoxia	7	22.29 ± 5.60	0.01	0.04

Data are expressed as mean percent ruthenium red paracellular permeability ± SE

^aMice were placed in hypoxia, 7% O₂, for 4 hours

Figure 6.2. Representative electron photomicrographs of *in vivo* ruthenium red paracellular permeability in endometrial epithelial cells of CD73^{-/-}, A2BR^{-/-} and wild-type (C57BL/6) mice. Ruthenium red appears as a black electron dense deposit. Arrows indicate paracellular space. No ruthenium red is observed in this paracellular space from a C57BL/6 mouse. Ruthenium red was frequently observed in the paracellular spaces from both CD73^{-/-} and A2BR^{-/-} deficient mice. Electron photomicrographs magnification, 50,000X.

Figure 6.2

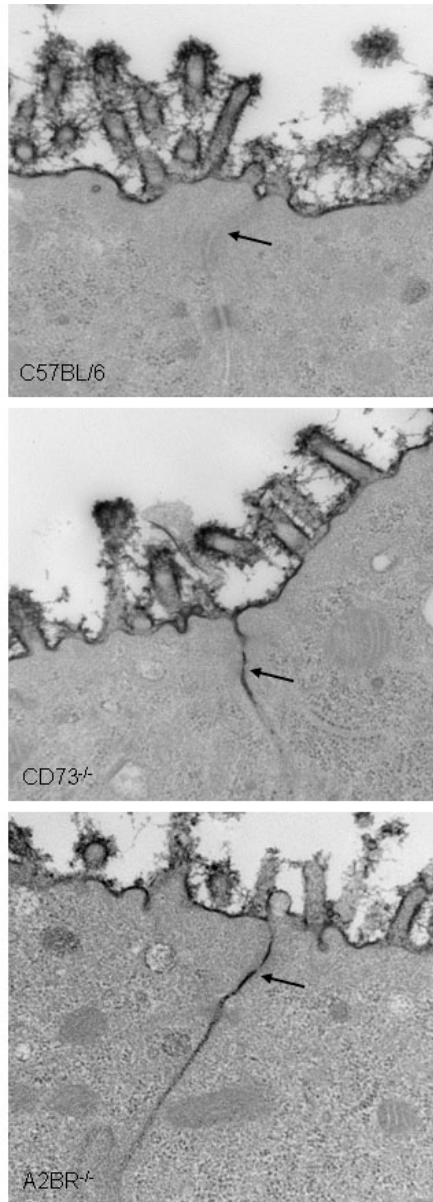


Table 6.2. Depth of ruthenium red paracellular permeability in endometrial epithelial cells of CD73^{-/-}, A2BR^{-/-}, and C57BL/6 mice

Mouse	N	Mean ± SE	p value (normoxia vs hypoxia)	p value (vs hypoxia C57BL/6)
C57BL/6 normoxia	6	978.57 ± 212.09		
^a C57BL/6 hypoxia	6	1050.00 ± 215.05	NS	--
CD73 ^{-/-} normoxia	6	1126.00 ± 133.35		
^a CD73 ^{-/-} hypoxia	6	1522.22 ± 304.62	NS	NS
A2BR ^{-/-} normoxia	7	1123.07 ± 361.64		
^a A2BR ^{-/-} hypoxia	7	1375.00 ± 330.71	NS	NS

Data are expressed as mean micron depth ± SE of ruthenium red paracellular permeability, measured from the apical surface to the deepest extent of ruthenium red fluxed down the paracellular space.

^aMice were placed in hypoxia, 7% O₂, for 4 hours

Barrier function in CD73^{-/-} mice can be rescued by intraperitoneal injection of 5'-N-ethylcarboxamidoadenosine (NECA), a stable analog of adenosine

To determine if the barrier protective role in endometrial epithelial cells requires adenosine generated by CD73, CD73^{-/-} mice were injected intraperitoneally (IP) with 5'-N-ethylcarboxamidoadenosine (NECA) 0.1mg/kg or 0.9% NaCl containing an equal volume of NECA vehicle, dimethylsulfoxide (DMSO, 0.006%) prior to 4 hour hypoxia exposure. NECA is a stable analog of adenosine that non-specifically activates adenosine receptor subtypes, including A2BR. The dosing and administration of NECA to CD73^{-/-} mice was identical to that previously described (205, 253). A significant reduction of ruthenium red paracellular permeability occurred with IP NECA injection (Figure 6.3 and Table 6.3). The ability to rescue barrier disruption in hypoxia conditioned CD73^{-/-} mice strongly suggest that adenosine is responsible for CD73's and A2BR's observed role in endometrial epithelial barrier function.

Figure 6.3. Barrier function in CD73^{-/-} mice following intraperitoneal injection of 5'-N-ethylcarboxamidoadenosine (NECA). CD73^{-/-} mice were intraperitoneally (IP) injected with 5'-N-ethylcarboxamidoadenosine (NECA) 0.1mg/kg or 0.9% NaCl containing an equal volume of NECA vehicle, dimethylsulfoxide (DMSO) 0.1ml/20g (DMSO content, 0.006%) prior to being exposed to hypoxia (7% O₂) for 4 hours. IP NECA caused a significant reduction of ruthenium red paracellular permeability in CD73^{-/-} mice. Error bars represent ± 1 SE. Mice per condition, n=5. Individual mice are represented by a single triangle. (*p<0.05)

Figure 6.3

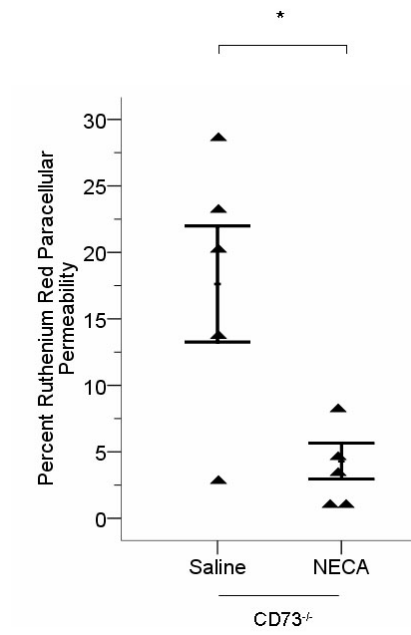


Table 6.3. Barrier function in CD73^{-/-} mice can be rescued by intraperitoneal injection of 5'-N-ethylcarboxamidoadenosine (NECA) – Data summary from Figure 6.3

CD73^{-/-}	N	Mean ± SE (%)	p value (saline vs NECA)
a Saline	5	17.6 ± 4.4	--
a NECA	5	4.2 ± 1.4	0.03

Data are expressed as mean percent ruthenium red paracellular permeability ± SE

aMice were placed in hypoxia, 7% O₂, for 4 hours

Summary, Sub-aim 2A

In specific aim 1, CD73 was significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs compared to well-differentiated and non-invasive ECs. This suggested that CD73's expression in normal endometrium might be related to some important aspect of normal endometrial epithelial cell function, such as barrier function. It is known that CD73-generated adenosine and adenosine's activation of A2BR induces endothelial and epithelial barrier function in response to cellular stressors. We therefore assessed whether CD73-generated adenosine may be important for epithelial barrier function in normal endometrium. In mice deficient for CD73 or A2BR, a significant loss of endometrial epithelial barrier function was observed when mice were stressed with hypoxia. Barrier function in hypoxia conditioned CD73^{-/-} mice was rescued by intraperitoneal injection of NECA, a stable analog for adenosine. These results suggest that CD73-generated adenosine and adenosine's activation of A2BR has an important role in maintaining epithelial barrier function in the normal endometrial epithelium. This follows the known mechanism of adenosine's barrier function (Stressor → CD73 → Adenosine → A2BR → Barrier Function).

Sub-aim 2A examined the central theme that adenosine's barrier function is a product of CD73's generation of adenosine and its activation of the adenosine receptor subtype, A2BR. Sub-aim 2B examines the central theme that adenosine's barrier function effect is supported by changes in the expression of purinergic pathway members to support the extracellular accumulation of adenosine. This central theme has been supported by previous studies demonstrating that cellular stress is associated with cytokine release or increased steady-state levels of HIF-1 α , which lead to the increased expression of members of the purinergic pathway important for the production (*CD39* and *CD73*) and activity (*A2BR*) of extracellular adenosine, while expression of members of the purinergic pathway that are important for the internalization of extracellular adenosine (ENTs) or its deamination (*ADA*) are concomitantly down-regulation. Overall, these events support the accumulation and activity of adenosine at the extracellular surface.

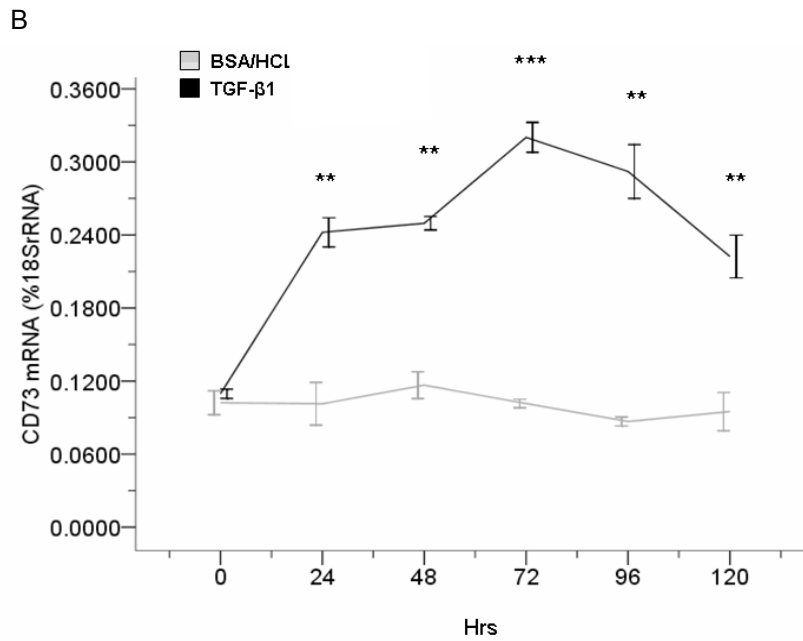
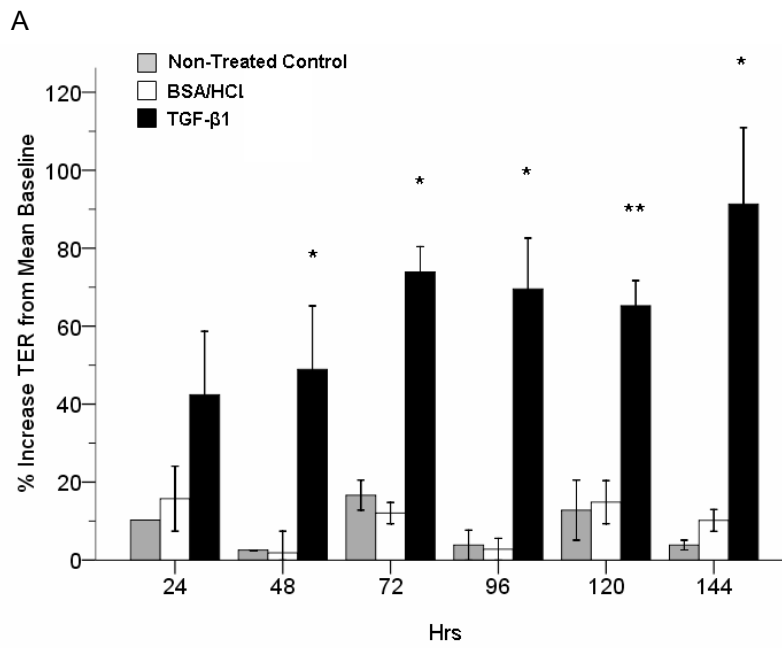
Transforming growth factor- β 1 (TGF- β 1) increases transepithelial resistance and CD73 expression in endometrial carcinoma cells, HEC-1A

TGF- β s are ubiquitous cytokines that are multifunctional, and dependent on cell type, regulate cell proliferation and differentiation, angiogenesis, immune actions, steroidogenesis, and events central to tissue remodeling and repair (reviewed, (254). TGF- β 1 has been established to increase barrier function in intestinal epithelial cells (188-193). Interestingly, TGF- β 1's barrier function may be co-dependent on its induction of a cAMP-mediated barrier function (190). cAMP is a primary secondary messenger of A2BR (179). A direct association of TGF- β 1's barrier function and adenosine signaling has not been reported, however. We therefore assessed TGF- β 1's ability to induce CD73 in an *in vitro* system. TGF- β 1 can induce growth inhibition in carcinoma cells (tumor suppressor function) or EMT (tumor promotive function). TGF- β 1's ability to increase barrier function in HEC-1A cells was first assessed. TGF- β 1 treatment

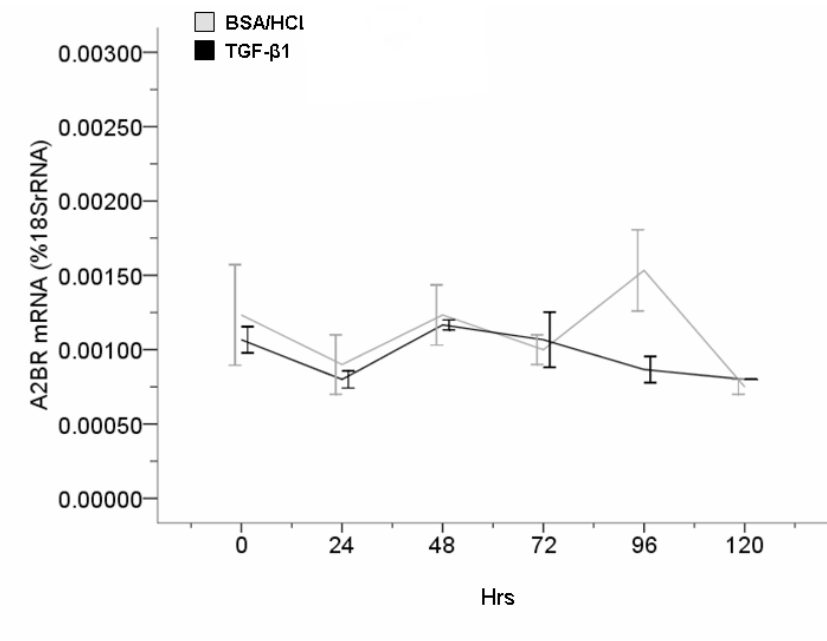
(2.5ng/ml) significantly increased transepithelial resistance (TER) in HEC-1A monolayers with increasing time (TGF- β 1: 48 Hrs, mean $43.48 \pm 10.80\%$; 72 Hrs, mean $67.40 \pm 7.50\%$; 96 Hrs, mean $63.03 \pm 9.96\%$; 120 Hrs, mean $58.70 \pm 7.50\%$; 144 Hrs, mean $84.76 \pm 13.06\%$) (Figure 6.4 A). Transepithelial resistance (TER) is an electrophysiological measure of the resistance of the paracellular pathway within an epithelial monolayer. Increased TER is an indication of increased barrier function. These findings suggest that TGF- β 1's activity in HEC-1A cells is tumor suppressive. This is supported by studies of prolonged TGF- β 1 treatment (144 hours), 2.5ng/ml (255) or 10ng/ml (256), that report TGF- β 1 to suppress growth of HEC-1A cells. Likewise, migration and invasion rates for HEC-1A cells are unaffected following 48 hours of TGF- β 1 treatment (255). Similar to TER, with increasing time of TGF- β 1 treatment, TGF- β 1 significantly increased *CD73* transcripts (Figure 6.4 B). *A2BR* transcripts were not affected by TGF- β 1, and *CD73* transcripts did not increase in a similar fashion (early time points) in TGF β R1 deficient (222) Ishikawa cells, an endometrial carcinoma cell line (Figure 6.4 C-D). Increased *CD73* transcript expression was associated with increased CD73 protein following 72 hours of TGF- β 1 treatment (Figure 6.4 E and Table 6.4).

Figure 6.4. Transforming growth factor- β 1's effects on transepithelial resistance (TER) and CD73 expression in endometrial carcinoma cells, HEC-1A. (A) Barrier function of HEC-1A cells treated with 2.5ng/ml of TGF- β 1 or an equal volume of the vehicle control, 1mg/ml bovine serum albumin/4mM hydrochloric acid (BSA/HCl) was determined by measuring transepithelial resistance (TER). TGF- β 1 significantly increased HEC-1A TER over BSA/HCl treatment at 48, 72, 96, 120, and 144 hours. (B) and (C) qRT-PCR of TGF- β 1 (2.5ng/ml) and BSA/HCl treated HEC-1A cells. (B) CD73 transcripts were significantly increased by TGF- β 1 treatment. (C) A2BR transcripts were not affected by TGF- β 1. (D) CD73 transcripts are not induced at the earlier time points by TGF- β 1 (2.5ng/ml) treatment of Ishikawa cells, which are deficient in TGF β R1. (E) Representative images of CD73 immunofluorescence following 72 hours of TGF- β 1 (2.5ng/ml) or BSA/HCl treatment of HEC-1A cells. TGF- β 1 or BSA/HCl was added to HEC-1A monolayers every 24 hours. Error bars represent \pm 1 SE. (* p <0.05, ** p <0.005, *** p <0.0005). Image magnification 20X

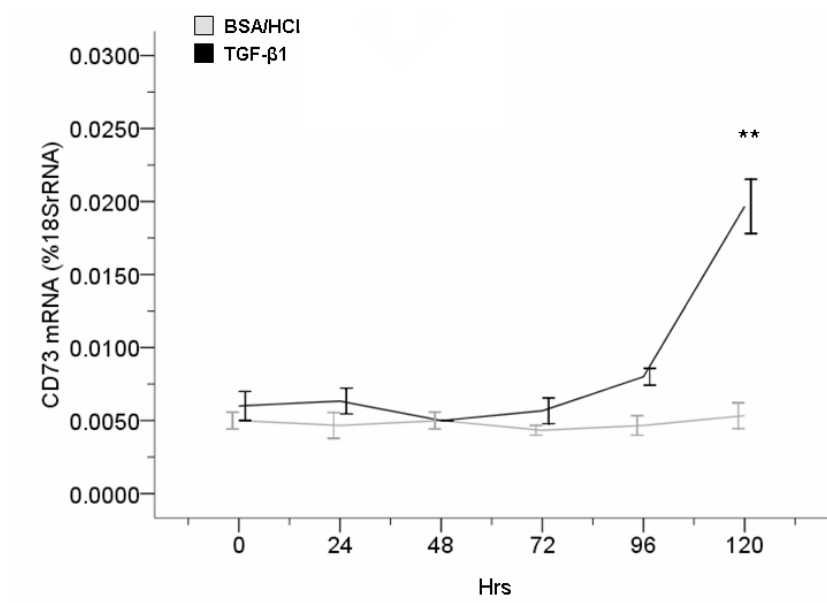
Figure 6.4



C



D



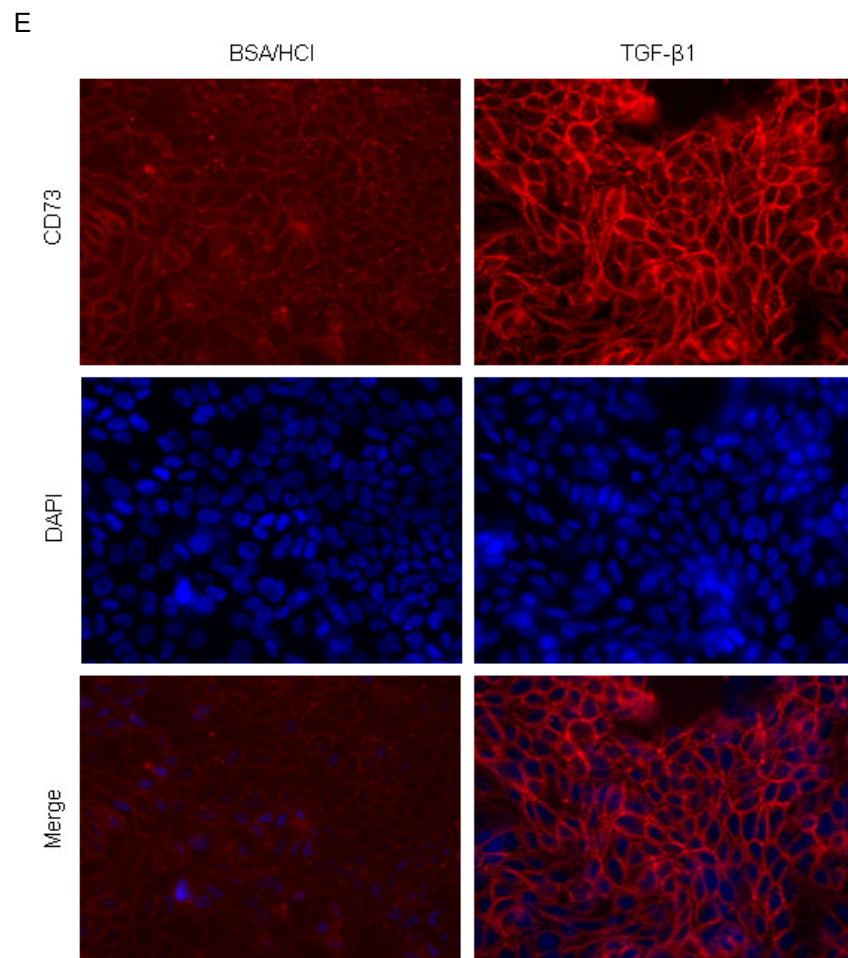


Table 6.4. Relative fluorescence intensity of CD73 in transforming growth factor- β 1 (TGF- β 1) and vehicle treated endometrial carcinoma cells, HEC-1A

Hours	N	^aMean Intensity \pm SE X10,000	p value (BSA/HCl vs TGF-β1)
0			
BSA/HCl	3	350.78 \pm 19.92	--
TGF- β 1	3	375.10 \pm 22.61	NS
24			
BSA/HCl	3	356.19 \pm 21.70	--
TGF- β 1	3	615.02 \pm 54.02	0.0003
48			
BSA/HCl	3	321.61 \pm 26.01	--
TGF- β 1	3	593.87 \pm 46.04	0.0001
72			
BSA/HCl	3	318.19 \pm 18.13	--
TGF- β 1	3	715.28 \pm 28.31	0.0000001

^aData are expressed as relative mean \pm SE fluorescence intensity of CD73 from 3 independent studies. Relative fluorescence intensity of CD73 was determined by capturing ten 20X images per treatment group/time interval and assessing integrated density by NIH Image J software.

TGF- β 1's induction of CD73 occurs through the canonical Smad-mediated signaling pathway.

TGF- β -induced signaling is mediated by Smad-dependent (canonical) and -independent (non-canonical) pathways. Non-canonical signaling pathways from TGF- β 1's activation of TGF- β receptors include Rho-p160Rock, TAK1-p38-MAPK, and Ras-Raf-Mek-Erk-MAPK (reviewed, (257)). TGF- β 1's induction of barrier function in intestinal epithelial cells is an event that occurs through TGF- β 's Smad-mediated signaling pathway (192). To examine whether TGF- β 1 treatment of HEC-1A cells was associated with the induction of canonical Smad-mediated signaling, HEC-1A cells were treated with TGF- β 1 (2.5ng/ml) and assessed for Smad2 phosphorylation. With TGF- β 1 treatment increased levels of Smad2 phosphorylation at serines 465 and 467 was found, indicating activation of TGF β R1 and Smad-mediated signaling (258-260) (Figure 6.5). E-cadherin levels did not change with TGF- β 1 treatment even at extended time points, which strongly suggests that HEC-1A cells do not undergo EMT with TGF- β 1 treatment. Similar studies have shown that E-cadherin remains membrane-bound with identical TGF- β 1 treatment time points in HEC-1A cells (255). For TGF- β 's canonical signaling to induce gene transcription, a heteromeric complex involving phosphorylated Smad2 and Smad3 and a co-Smad member, Smad4, must assemble (261-263). To determine whether TGF- β canonical signaling mediates CD73 expression, the cell-permeable pyrrolopyridine compound, SIS3 was used. SIS3 selectively inhibits the phosphorylation of Smad3 to prevent the assembly of the Smad2, 3, and 4 complex which is required for TGF- β -directed gene transcription. Following 12 and 24 hours of treatment, 1 μ M SIS3 significantly inhibited the increase of *CD73* transcripts typically induced by TGF- β 1 in HEC-1A cells (Figure 6.6 A). SIS3 treatment also led to a decrease in CD73 protein expression. Representative images following 24 hours of TGF- β 1 (2.5ng/ml) and DMSO (SIS3 vehicle) or TGF- β 1 (2.5ng/ml) and SIS3 (1 μ M) treatment are shown in Figure 6.6 B. These studies indicate that CD73 is induced by TGF- β 's canonical, Smad-mediated signaling pathway.

Figure 6.5. TGF- β canonical, Smad-mediated signaling in endometrial carcinoma cells, HEC-1A. HEC-1A cells were treated with 2.5ng/ml of TGF- β 1 or an equal volume of the vehicle control, 1mg/ml bovine serum albumin/4mM hydrochloric acid (BSA/HCl), and assessed by immunoblotting for TGF- β canonical, Smad-mediated signaling. TGF- β 1 increased Smad2 phosphorylation at serines 465 and 467, indicating TGF β R1 activation and Smad-mediated signaling. E-cadherin levels did not change, indicating HEC-1A cells do not undergo EMT with TGF- β 1 treatment.

Figure 6.5

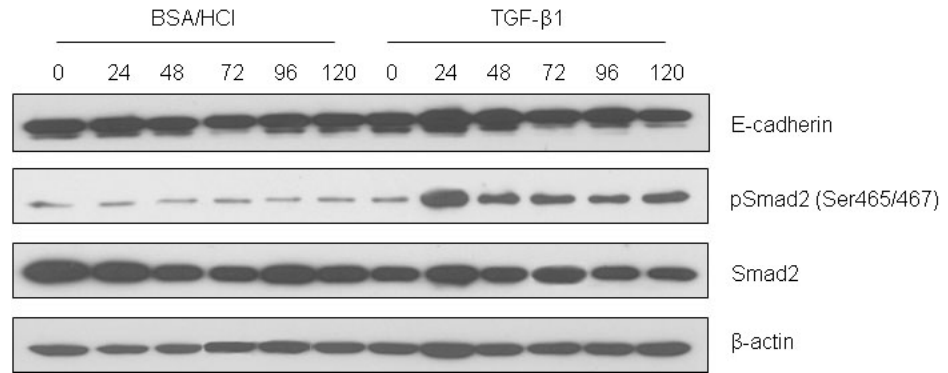
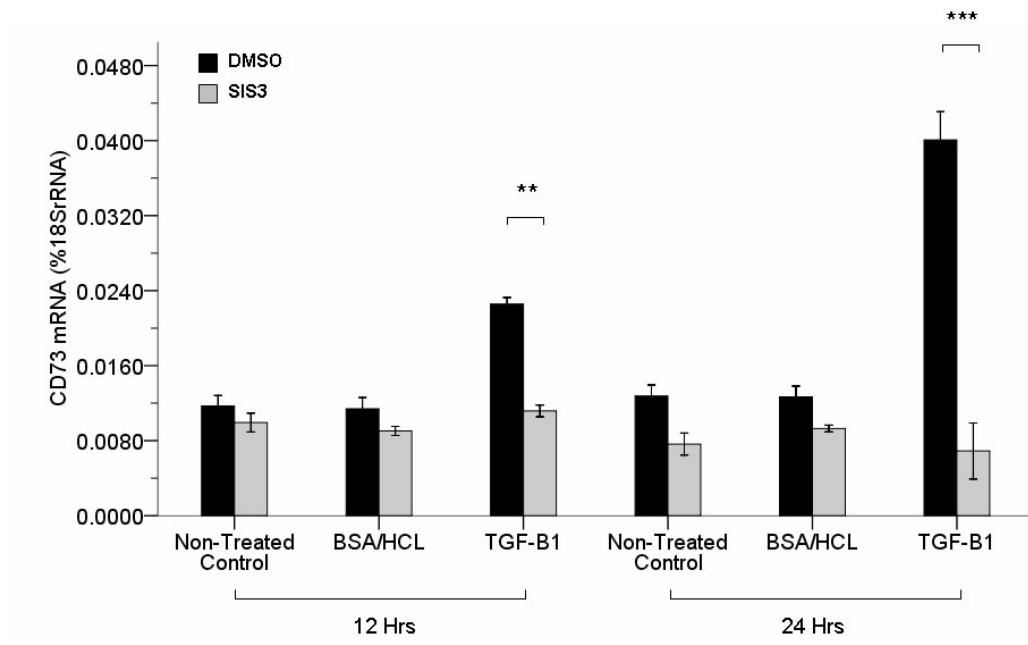
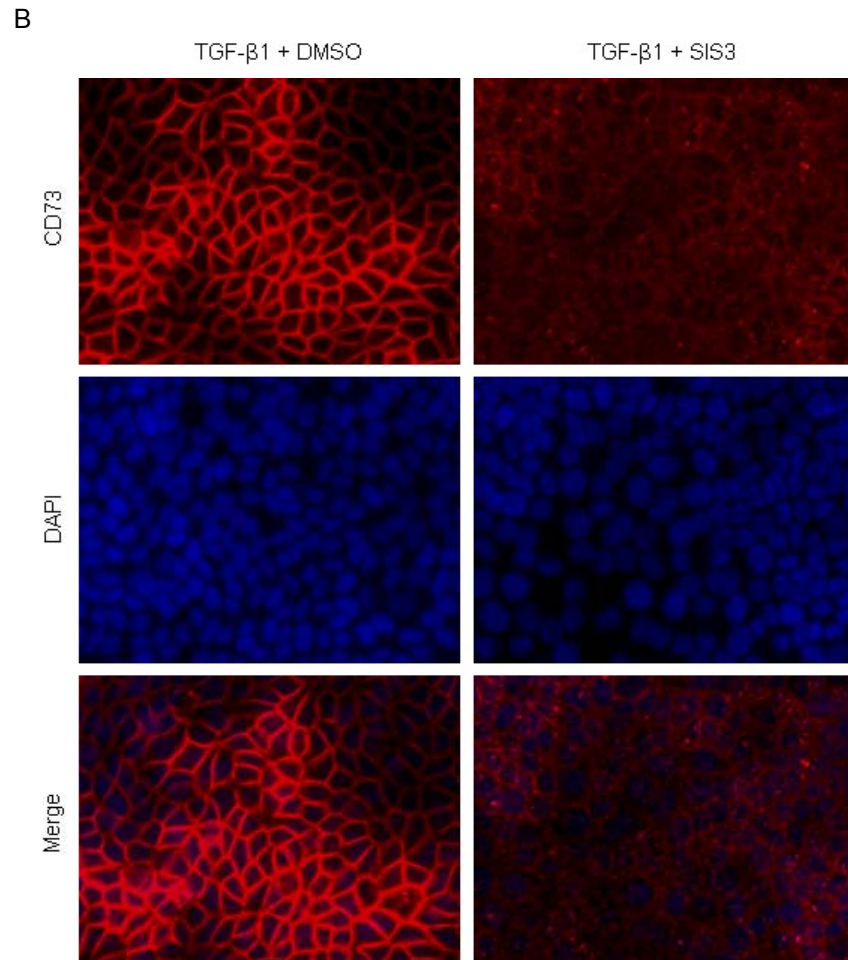


Figure 6.6. CD73 expression in endometrial carcinoma cells, HEC-1A, following SIS3 inhibition of TGF- β canonical, Smad-mediated signaling pathway. HEC-1A cells were treated with 2.5ng/ml of TGF- β 1 or an equal volume of the vehicle control, 1mg/ml bovine serum albumin/4mM hydrochloric acid (BSA/HCl), and 0.001% DMSO or 1 μ M of the Smad3 inhibitor, SIS3. (A) Following 12 and 24 hours of treatment, SIS3 significantly inhibits TGF- β 1's increase of *CD73* transcripts measured by qRT-PCR. (B) Representative images of CD73 immunofluorescence following 24 hours of TGF- β 1 (2.5ng/ml) and DMSO or TGF- β 1 (2.5ng/ml) and SIS3 (1 μ M) treatment of HEC-1A cells. Error bars represent \pm 1 SE. (* p <0.05, ** p <0.005, *** p <0.0005). Image magnification 20X

Figure 6.6

A





Induction of *CD73* and *A2BR* expression by hypoxia in the endometrial carcinoma cells, HEC-1A and HEC-1B, and cervical carcinoma cells, WISH-HeLa

In hypoxia, *CD73* and *A2BR* expression is induced as much as 6- (204) to 12-fold (210) and 5-fold (210), respectively, over levels seen during normoxia. Increased steady-state levels of HIF-1 α in conditions of hypoxia are central to the gene expression induced by HIF-1. The regulation of HIF-1 α expression and activity *in vivo* occurs at multiple levels, including mRNA expression, protein expression, nuclear localization, and transactivation (reviewed, (252)). Among these, the most intensively studied has been the regulation of steady-state HIF-1 α protein levels. Under normoxic conditions, HIF-1 α is ubiquitinated and subject to proteasomal degradation by von Hippel-Lindau (VHL), a E3 ubiquitin-protein ligase (264-268). HIF-1 binding sequences in *CD73* and *A2BR* promoters have been described, and HIF-1 directly induces *CD73* (204) and *A2BR* (210) expression. Hypoxia is a characteristic feature of most carcinomas, including endometrial carcinoma (269-272). HIF-1 targets genes that are particularly relevant to cancer progression, including those involved in angiogenesis, glucose transport and glycolytic enzymes, genes involved in survival, and genes important to cancer cell invasion (reviewed, (273)). Because HIF-1 induces *CD73* and *A2BR* to increase extracellular generation of adenosine and adenosine's induction of barrier function in normal tissues stressed by hypoxic, it is possible that carcinoma cells have selectively lost the ability to induce *CD73* and *A2BR* by HIF-1. This event would be hypothesized to be important, as maintaining barrier function is counterintuitive to the progressive of carcinoma cells with hypoxia. Therefore, we assessed the hypoxia-mediated induction of *CD73* and *A2BR* in endometrial carcinoma cell lines, HEC-1A and HEC-1B, and in the cervical carcinoma cell line, WISH-HeLa. Figure 6.7 shows the increased steady-state levels of HIF-1 α in HEC-1A (A), HEC-1B (B), and WISH-HeLa (C) cells with hypoxia (1% O₂, 5% CO₂). These data suggest the potential for HIF-1 to induce gene expression in these carcinoma cells. Using qRT-PCR, *CD73* and *A2BR* expression was measured in HEC-1A, HEC-1B, and

WISH-HeLa cells exposed to normoxic or hypoxic (1% O₂, 5% CO₂) conditions for various time periods. Interestingly, *CD73* and *A2BR* transcripts were unchanged by hypoxia in HEC-1A cells (Figure 6.8 A-B). In HEC-1B cells, *A2BR* transcripts significantly increased at 6, 12, and 24 hours of hypoxia (Figure 6.8 D). *CD73* transcripts levels were unchanged with hypoxia in HEC-1B cells (Figure 6.8 C). In WISH-HeLa cells, both *CD73* and *A2BR* transcripts were induced by hypoxia at 24 hours (*CD73*) and 4, 6, 12, and 24 hours (*A2BR*) (Figure 6.8 E-F). These results indicate that adenosine-induced barrier function in hypoxic endometrial carcinoma cells is prevented by inhibiting HIF-1's induction of *CD73* and/or *A2BR* (HEC-1A).

Figure 6.7. Immunoblots of steady-state HIF-1 α in endometrial carcinoma cells, HEC-1A and HEC-1B, and cervical carcinoma cells, WISH-HeLa, in hypoxia. HEC-1A (A), HEC-1B (B) and WISH-HeLa (C) cells were exposed to normoxia or hypoxia (1% O₂, 5% CO₂) for indicated times and steady-state levels of HIF-1 α determined by immunoblots. Expression of HIF-1 α , with hypoxia exposure, was found in HEC-1A, HEC-1B, and WISH-HeLa cells.

Figure 6.7

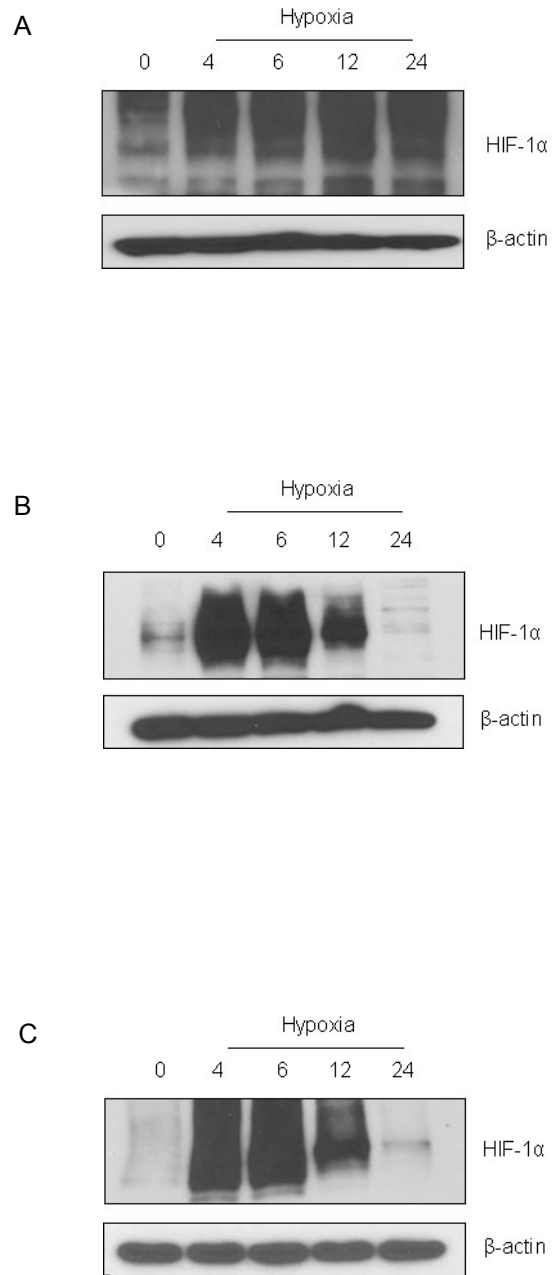
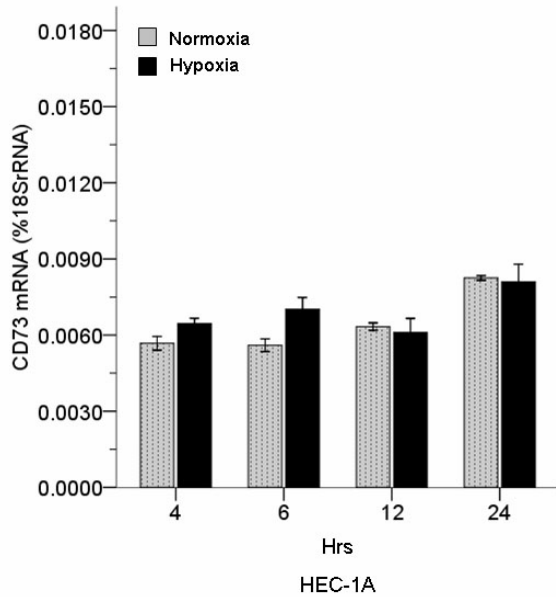


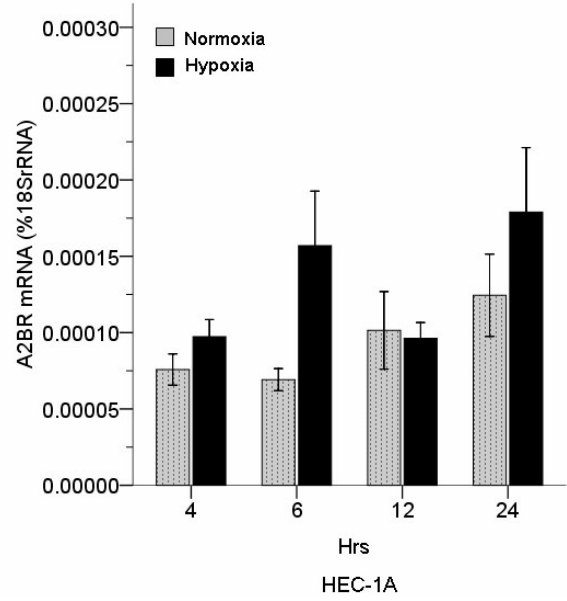
Figure 6.8. *CD73* and *A2BR* expression in endometrial carcinoma cells, HEC-1A and HEC-1B, and cervical carcinoma cells, WISH-HeLa, in normoxia and hypoxia. HEC-1A (A-B), HEC-1B (C-D) and WISH-HeLa (E-F) cells were exposed to normoxia or hypoxia (1% O₂, 5% CO₂) for indicated times and *CD73* and *A2BR* expression assessed by qRT-PCR. An increase in *CD73* or *A2BR* transcripts in HEC-1A cells did not occur with hypoxia (A-B). *A2BR* transcripts in hypoxic HEC-1B cells significantly increased at 6, 12, and 24 hours as compared to normoxic cells at similar time points (D). *CD73* transcripts did not increase with hypoxia in HEC-1B cells (C). (E) *CD73* transcripts in hypoxic WISH-HeLa cells significantly increased at 24 hours compared to normoxic cells. (F) *A2BR* transcripts were significantly increase at 4, 6, 12, and 24 hours in hypoxic WISH-HeLa cells. Transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of transcript/molecules of *18S* rRNA (%18SrRNA). Error bars represent ± 1 SE. (*p<0.05)

Figure 6.8

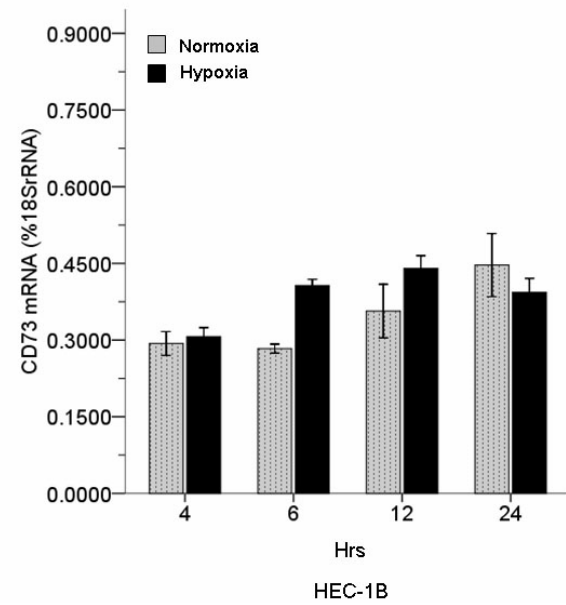
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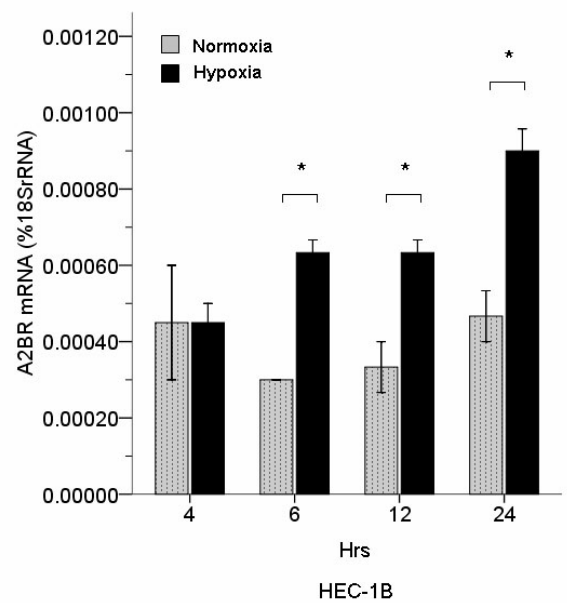
B

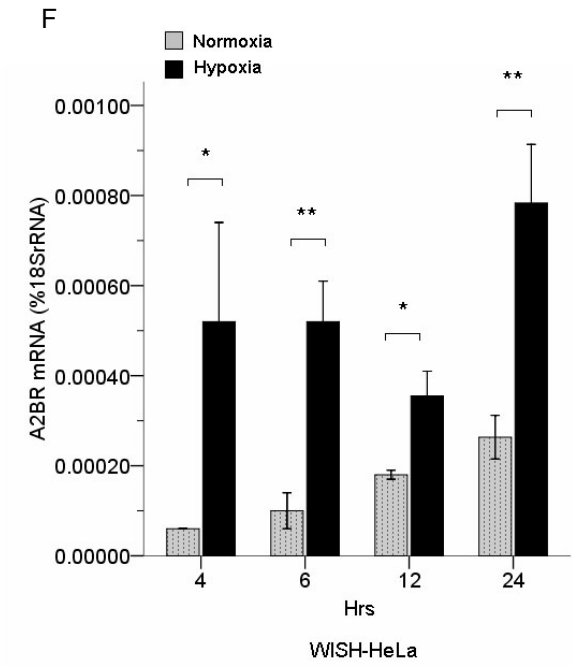
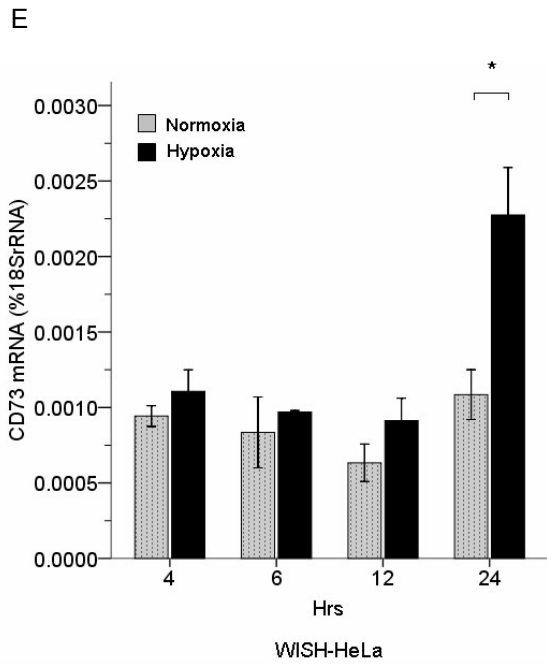


C



D





Summary – Sub-aim 2B

Sub-aim 2A demonstrated that CD73-generated adenosine and A2BR are important for endometrial epithelial barrier function. This is consistent with the central theme that stressors, such as hypoxia, to induce the production of adenosine and adenosine's barrier function by CD73 activity. Another important role of adenosine mediated barrier function (second central theme) involves the increased production and activity of adenosine at the extracellular surface of the cell. This involves the increased expression of members of the purinergic pathway important for the production (*CD39* and *CD73*) and activity (*A2BR*) of extracellular adenosine, with concurrent down-regulation of members of the purinergic pathway that are important for the internalization of extracellular adenosine (ENTs) or its deamination (*ADA*). This was the focus of sub-aim 2B and its studies. Events caused by stressors, such as cytokine release with inflammation and increased steady-state levels of HIF-1 α with hypoxia, are two important events that lend to the increased expression of *CD73* and/or *A2BR*. Inflammation and hypoxia are persistent stressors of most carcinoma types, including EC. The cytokine, TGF- β 1, was found to induce CD73 by the TGF- β canonical, Smad-mediated signaling pathway. It was also found that TGF- β 1 induced barrier function in these well-differentiated EC cells. TGF- β 1 acts as a suppressor of carcinoma progression in early stage disease. However, becomes a supporting feature for carcinoma progression in late stage disease. These data raise questions as to whether the barrier function activities of TGF- β and CD73 are one in the same, and whether TGF- β 1's induction of CD73 and its adenosine-mediated barrier function is one of the tumor suppressive events of TGF- β signaling in early disease. These data are the first to describe a relationship between TGF- β 1 and CD73 in non-leukocyte cells. Data involving the increased expression of *CD73* and *A2BR* by hypoxia suggested that adenosine-induced barrier function in hypoxic endometrial carcinoma cells is prevented primarily by inhibiting HIF-1's induction of *CD73*. Induced expression of *A2BR* was significant in HEC-1B and WISH-HeLa cells, however for HEC-1B increased levels of CD73 did

not occur. This data taken together suggest that inhibiting the induced expression of *CD73*, which generally occurs with hypoxia, is sufficient enough to prevent increased adenosine production by these carcinoma cells, and thus the induction of barrier function.

CHAPTER SEVEN

Results

Specific Aim 3. Demonstrate that CD73's pattern of expression and catalytic activity are consistent with the proposed role of CD73 in the maintenance of the epithelial phenotype.

Introduction

Adherens junction protein, E-cadherin, is the most recognized cell-cell adhesion protein that is lost in metastatic carcinomas. E-cadherin's ability to induce cell-cell adhesions in transformed mesenchymal cells and its initiation of cell-cell contacts and adhesion development has earned E-cadherin the distinction as being the essential caretaker of the epithelial phenotype (274). However, the formation, establishment, maintenance, and regulation of cell-cell adhesions are a dynamic process dependent on the presence and coordination of many cell-cell adhesion proteins and their associated counterparts. As introduced in this dissertation's General Introduction, the aberrant localization or loss of expression of various, non-E-cadherin ("other") cell-cell adhesion proteins or their regulators can impact a wide variety of events, including proliferation and transcriptional control, basolateral receptor activity, polarity, migration and invasion, and therapeutic sensitivity. These effects indicate that "other" cell-cell adhesion proteins have significant importance to the epithelial phenotype and carcinoma progression and metastasis. Therefore, it can be argued that many other proteins, and not just E-cadherin, involved in the dynamics of cell-cell adhesions are caretakers of the epithelial phenotype.

In vivo studies in specific aim 2 strongly support that CD73-generated adenosine is important for endometrial epithelial barrier function. Barrier function is a homeostatic event whose actions are directly related to the regulation and maintained integrity of cell-cell adhesions and their individual proteins. In the initial steps of the metastatic cascade, carcinoma cells will shed their prototypical epithelial characteristics, including cell-cell adhesions. This action

provides carcinoma cells a phenotype that is more influencing of metastatic behavior. In specific aim 1, CD73 expression was significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs. The loss of cell-cell adhesion proteins, including E-cadherin (91-94) and occludin (95), is typical among these ECs. We believe that CD73's maintenance of the epithelial barrier and therefore its regulation of cell-cell adhesions is the contributing reason for CD73's loss of expression in moderately- and poorly-differentiated, invasive, and metastatic ECs. For this reason, we considered that if CD73 is central to the regulation of cell-cell adhesions, and thus a caretaker of the epithelial phenotype, then its expression in carcinoma cells would likely be lost with the transition from an epithelial to a mesenchymal phenotype. Such a loss of expression is characteristic of proteins that are involved in maintaining the epithelial phenotype.

This specific aim was intended to establish the pattern of expression and specific activity of CD73 in relation to the characteristic change of phenotypes seen in progressive and metastatic carcinomas. To accomplish this specific aim, cryosections of normal endometrium and ECs of well- and poorly-differentiated histotypes as well as endometrial carcinoma cell lines that were representative of varying EMT phenotypes (epithelial-like, epithelial and mesenchymal-like, and mesenchymal-like) were examined. CD73 expression was also assessed in the cervical carcinoma cell sub-line, WISH-HeLa, which spontaneously transitions from a mesenchymal to epithelial phenotype with growth confluency.

CD73 expression in proliferative and secretory phase and hormone inactive endometrium is significantly higher in epithelial cells

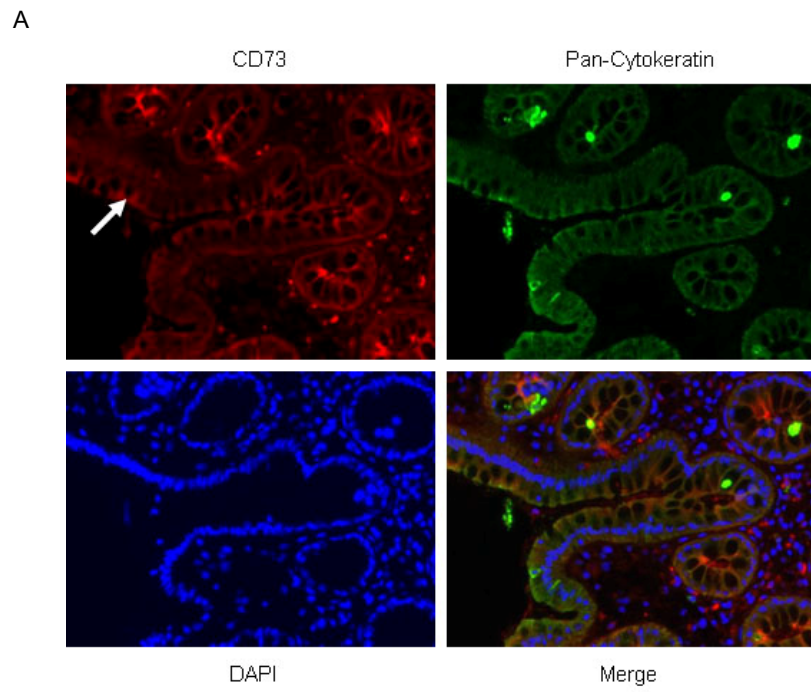
Studies in specific aim 2 show that CD73-generated adenosine and A2BR activation are involved in inducing barrier function in epithelial cells of the endometrium. Studies assessing CD73's generation of adenosine and its induction of barrier function in endothelial cells indicate that endothelial-expressed CD73 is largely responsible for the generation of adenosine that induces endothelial barrier function (196). We therefore would hypothesize that epithelial cells of the endometrium would exhibit higher levels of CD73 expression relative to other resident cell types. CD73's expression among the cellular components of human endometrium has not been previously characterized. To investigate the cell type expression pattern of CD73 in the normal endometrium, cryosections from proliferative and secretory phase and hormone inactive endometrium were double labeled with CD73 and pan-cytokeratin. Pan-cytokeratin served as an indicator of epithelial cells. Normal colon was used as a control reference for CD73, as CD73's expression pattern in normal colon has been described (275). CD73 is a glycosyl phosphatidylinositol (GPI) linked ecto-enzyme that is estimated to be 6-fold higher on the apical membrane of colonic crypt epithelium, as opposed to the basolateral membrane (275). Additionally, epithelial expression is higher than that of the surrounding stroma (275). Similar patterns of expression were observed in our studies when assessing CD73 in normal colon (Figure 7.1 A). These studies validated the CD73 antibody selected and used for the following studies.

In normal endometrium (Figure 7.1 B) a similar pattern of expression for CD73 was seen that had been described and observed within normal colon. CD73 was highly expressed in glandular epithelium of proliferative and secretory phase and hormone inactive endometrium. Stromal cells did express CD73, albeit at a lower intensity. Relative fluorescence intensities for CD73 in epithelial cells versus that of stromal cells are summarized in Table 7.1, and this data demonstrates that higher expression of CD73 indeed occurs in glandular epithelial cells. Similar

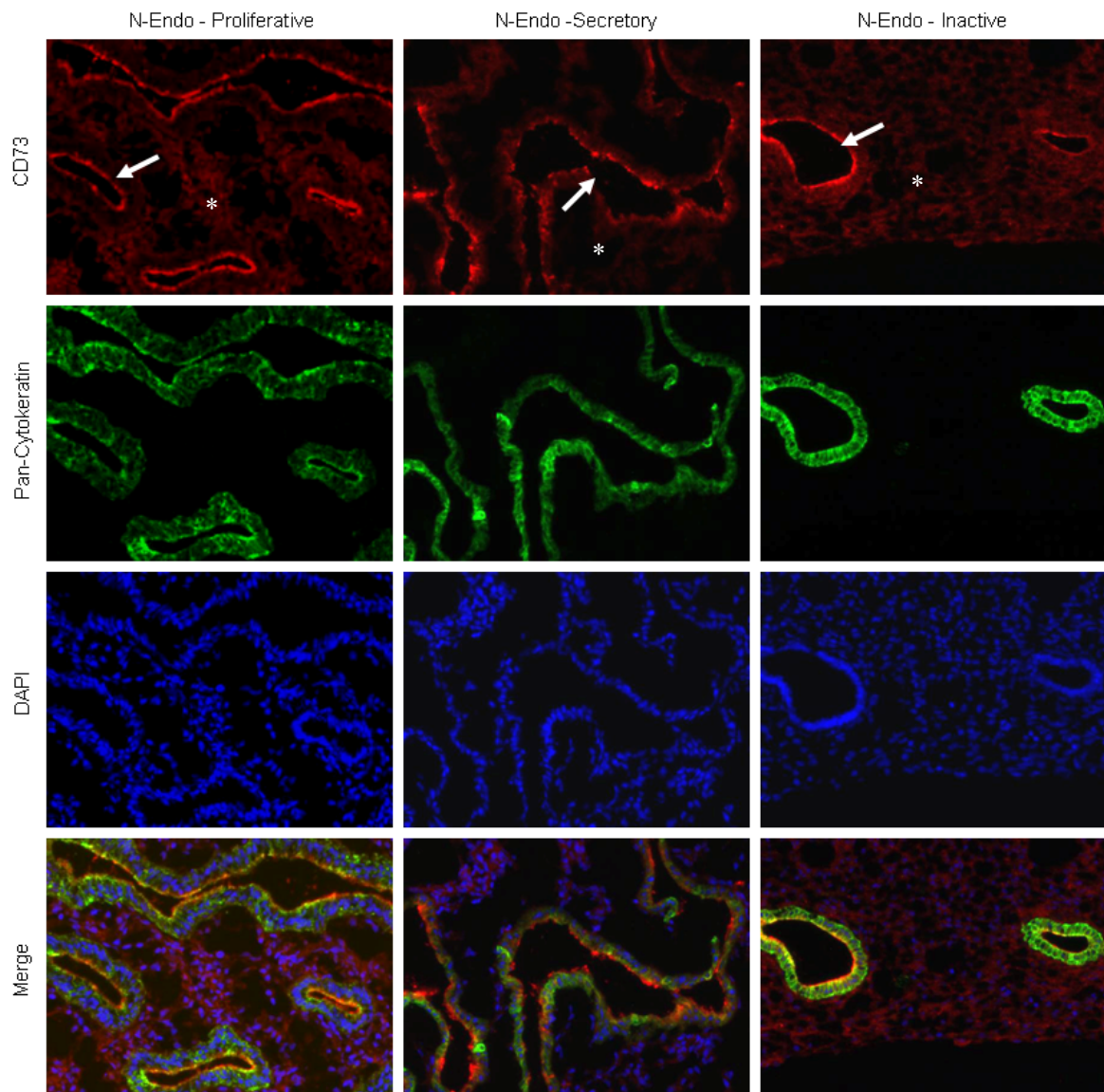
to that described and observed within the normal colon, CD73 was found in normal endometrium to be present at the apical and basolateral membranes, with apical intensity being higher than that observed for basolateral (Figure 7.1 C). The significant levels of CD73 expression identified in epithelial cells support the premise that CD73-generated adenosine production mediates barrier function in epithelial cells of the endometrium.

Figure 7.1. CD73 and pan-cytokeratin immunofluorescence in proliferative phase, secretory phase, and hormone inactive endometrium. Cryosections of normal colon and endometrium were characterized for CD73 expression by immunofluorescence. Normal colon served as a positive control. Pan-cytokeratin served as a marker of glandular epithelial cells. (A) In normal colon, membranous CD73 is present in epithelial colonic crypts (arrow). CD73 expression is also present, at a lower visible intensity, in surrounding stromal cells (asterisk). (B) CD73 was highly expressed in glandular epithelium (arrows) of proliferative phase, secretory phase, and hormone inactive endometrium. Stromal cells expressed CD73, but at a lower intensity. (C) CD73 is present at the apical and basolateral membranes, with apical intensity of CD73 being visibly higher than basolateral. Image magnification A and B, 20X; C, 40X.

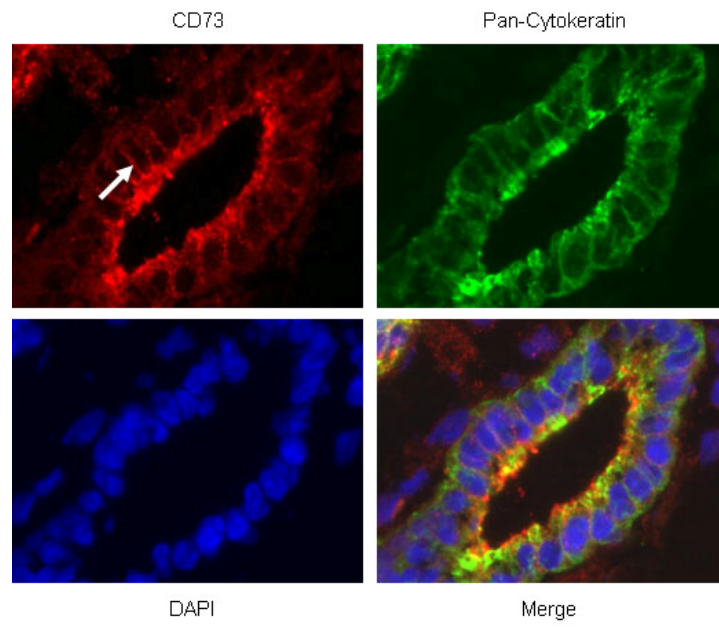
Figure 7.1



B



C



CD73's intensity of expression is retained in well-differentiated endometrial carcinomas, however lost in poorly-differentiated histotypes

Epithelial-to-mesenchymal transition (EMT) is characterized by the gain of expression of mesenchymal markers, such as S100A4 and vimentin, and the loss of expression of epithelial markers, such as E-cadherin and occludin. Well-differentiated ECs, such as Grade 1 endometrioid endometrial carcinoma (G1 EEC), typically maintain E-cadherin expression and are negative for S100A4 expression (255, 276). Poorly-differentiated EC histotypes, Grade 3 endometrioid endometrial carcinoma (G3 EEC) and uterine papillary serous carcinoma (UPSC), generally express the mesenchymal marker S100A4 and show absence for E-cadherin expression (255, 276). To investigate the pattern of CD73 expression in ECs with a phenotype either being more epithelial-like (well-differentiated, G1 EEC) or more mesenchymal-like (poorly-differentiated, G3 EEC or UPSC), cryosections of differing EC histotypes were double labeled for CD73 and pan-cytokeratin. Pan-cytokeratin served as an indicator of carcinoma cells. In G1 EEC, CD73 was expressed in carcinoma cells at a similar intensity to that seen in normal glandular epithelium (Figure 7.2 and Table 7.1). In carcinoma cells of poorly-differentiated histotypes, G3 EEC and UPSC, CD73 expression and intensity of expression was significantly lower than in G1 EEC and glandular epithelium expression in normal endometrium (Figure 6.2 and Table 6.1). These studies indicate that CD73's expression found to be down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs by qRT-PCR is a result of CD73's down-regulation in carcinoma cells. These data also support that the hypothesis that the loss of CD73 expression in poorly-differentiated histotypes is not a product of the overall loss of stromal cells, which occurs with these EC histotypes. Rather, it is the down-regulation of CD73 in carcinoma cells that appears to have a significant role in the progression of EC.

Figure 7.2. CD73 and pan-cytokeratin immunofluorescence in Grade 1 and Grade 3 endometrioid endometrial carcinoma (G1 EEC and G3 EEC) and uterine papillary serous carcinoma (UPSC). Cryosections of ECs of differing histotypes were characterized for CD73 expression by immunofluorescence. Pan-cytokeratin served as an indicator of carcinoma cells. In well-differentiated ECs, G1 EEC, CD73 is expressed in carcinoma cells (arrow) at a similar intensity to that seen in normal glandular epithelium (Table 7.1). Surrounding stroma was also positive for CD73 expression (asterisk), but stromal expression was typically weaker. In carcinoma cells of poorly-differentiated EC histotypes, G3 EEC and UPSC, CD73 expression and intensity of expression was significantly lower than that for G1 EEC. Image magnification 20X.

Figure 7.2

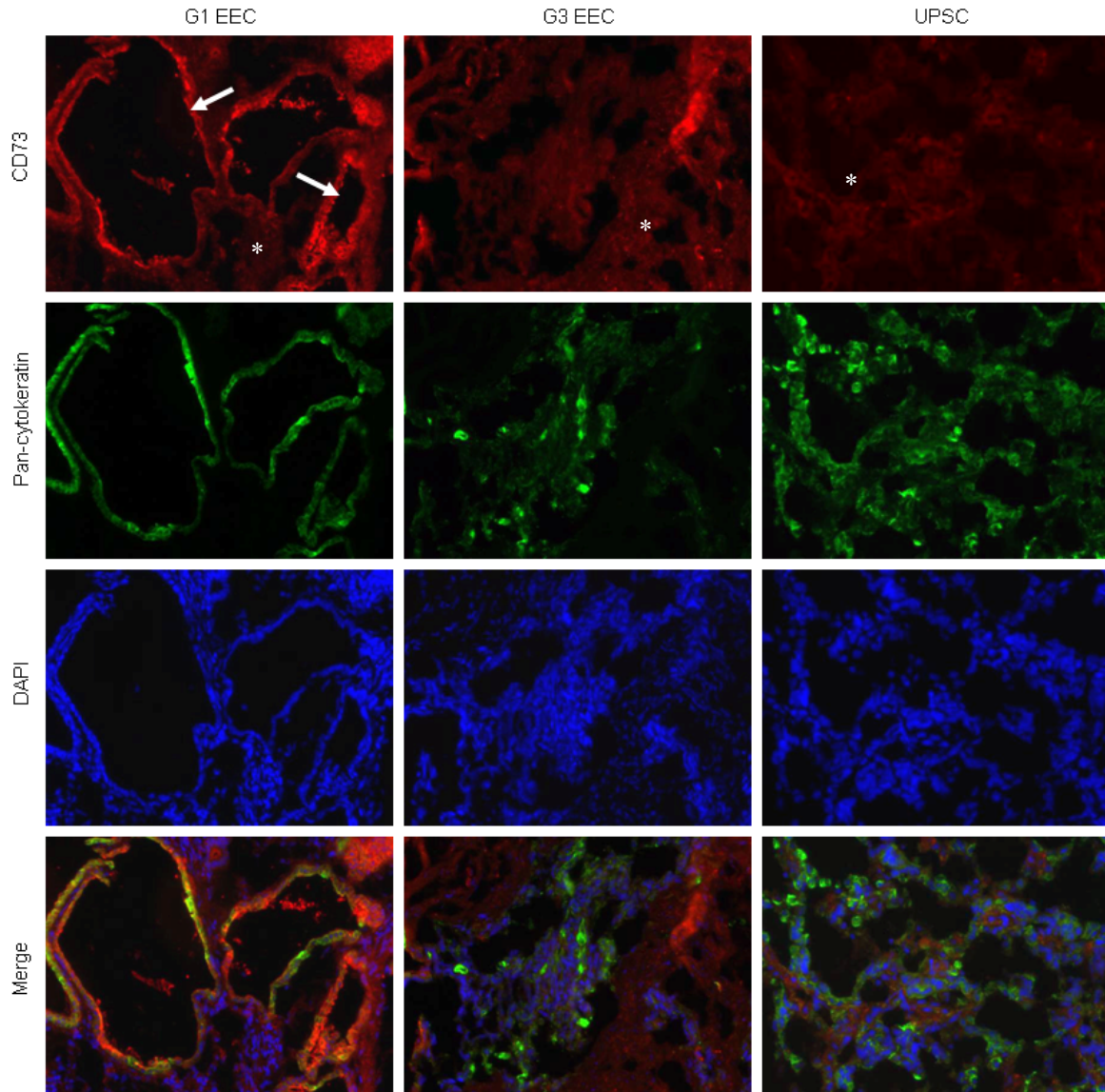


Table 7.1. Relative fluorescence intensity of CD73 in glandular epithelial cells and stromal cells in normal endometrium and endometrial carcinoma

	N	^a Mean ± SE	p value (epithelial vs stromal)	p value (N-Endo epithelial vs carcinoma)
N-Endo Proliferative				
Epithelial	2	1.68 ± 0.11	0.02	--
Stromal	2	1.00		
N-Endo Secretory				
Epithelial	2	1.91 ± 0.20	0.04	--
Stromal	2	1.00		
N-Endo Inactive				
Epithelial	2	1.62 ± 0.08	0.02	--
Stromal	2	1.00		
G1 EEC				
Carcinoma	2	2.03 ± 0.13	0.001	NS
Stroma/Connective Tissue	2	1.00		
G3 EEC				
Carcinoma	2	1.08 ± 0.05	NS	0.02
Stroma/Connective Tissue	2	1.00		
UPSC				
Carcinoma	2	1.10 ± 0.19	NS	0.04
Stroma/Connective Tissue	2	1.00		

^aData are expressed as mean fold fluorescence intensity of CD73 ± SE.

Stromal intensities were set at a baseline of 1.00 and intensity of glandular epithelial cells or carcinoma cells calculated as fold increase over stromal cells.

Relative fluorescence intensity of CD73 was determined by capturing four 20X images per tissue sample and assessing integrated density by NIH ImageJ software. A fixed area selection tool was

used to assess integrated density of glandular epithelial cells or carcinoma cells only and stromal cells only.

Abbreviations:

G1 EEC, Grade 1 endometrioid endometrial carcinoma; well-differentiated

G3 EEC, Grade 3 endometrioid endometrial carcinoma; poorly-differentiated

UPSC, uterine papillary serous carcinoma; poorly-differentiated

CD73 expression correlates with an epithelial-like phenotype

Epithelial-to-mesenchymal transition cannot be readily followed in time and space in human carcinomas. Therefore, much of the work describing the genetic re-programming that occurs with EMT and the phenotypic changes that are associated with this event have been characterized with *in vitro* studies. As previously discussed in the Introduction, loss of proteins regulating cell-cell adhesions can be associated with EMT. To further characterize whether loss of CD73 is associated with carcinoma cells shifting from an epithelial-like to a mesenchymal-like phenotype, as seen with many cell-cell adhesion proteins, CD73 expression was assessed in endometrial carcinoma cell lines that are representative of differing phases of EMT. These are indicated as epithelial-like, epithelial and mesenchymal-like, and mesenchymal-like. The term “like” is used here to emphasize that these carcinoma cell lines do not retain all characteristics central to an epithelial cell or a mesenchymal cell. Instead, they tend to retain qualities that are more epithelial (epithelial-like), a combination (epithelial and mesenchymal-like), or more mesenchymal (mesenchymal-like). These characteristics are represented in Figure 7.3. HEC-1A cells retain the classic cobblestone morphology of epithelial cells, express membranous E-cadherin, and have no expression of the mesenchymal marker, vimentin. HEC-1A cells are therefore considered epithelial-like. KLE is considered epithelial and mesenchymal-like for its cytoplasmic E-cadherin expression and simultaneous vimentin expression. AN3CA is considered mesenchymal-like with its spindle cell morphology and vimentin expression. AN3CA cells lack E-cadherin expression. *CD73* transcript levels were measured in various endometrial carcinoma cells that were representative of these three EMT categories. *CD73* transcript levels in epithelial-like endometrial carcinoma cells, HEC-1A and HEC-1B, were much higher than those observed in epithelial and mesenchymal-like endometrial carcinoma cells, KLE, Ishikawa, and ECC-1 (Table 7.2). *CD73* transcripts in the mesenchymal-like endometrial carcinoma cells, AN3CA, were undetectable (Table 7.2). *CD73* protein expression was apparent in the epithelial-like/well-

differentiated (cobblestone morphology) endometrial carcinoma cells, HEC-1A (Figure 6.4). CD73 protein expression was found in a few cells of the epithelial and mesenchymal-like EC cells KLE, and was absent in mesenchymal-like endometrial carcinoma cells, AN3CA. Importantly, the expression patterns of CD73 in these cell lines mirror that of well-differentiated G1 EEC (HEC-1A and HEC-1B) and the more poorly differentiated G3 EEC and UPSC (AN3CA), making these cell lines excellent tools for further *in vitro* studies.

Figure 7.3. Representative endometrial carcinoma cells with epithelial-like, epithelial and mesenchymal-like, and mesenchymal-like phenotypes. Hematoxylin and eosin stained and E-cadherin and vimentin immunofluorescence of endometrial carcinoma cells, HEC-1A, KLE, and AN3CA. HEC-1A cells retain characteristics classic of epithelial cells, including cobblestone morphology and membranous E-cadherin (red) expression. HEC-1A lack expression for the mesenchymal marker, vimentin (green). KLE cells have an epithelial and mesenchymal-like phenotype, as they simultaneously express cytoplasmic E-cadherin (red) and vimentin (green). AN3CA cells are mesenchymal-like, as cells show spindle morphology and express vimentin (green). AN3CA cells lack E-cadherin (red). Image magnification 40X.

Figure 7.3

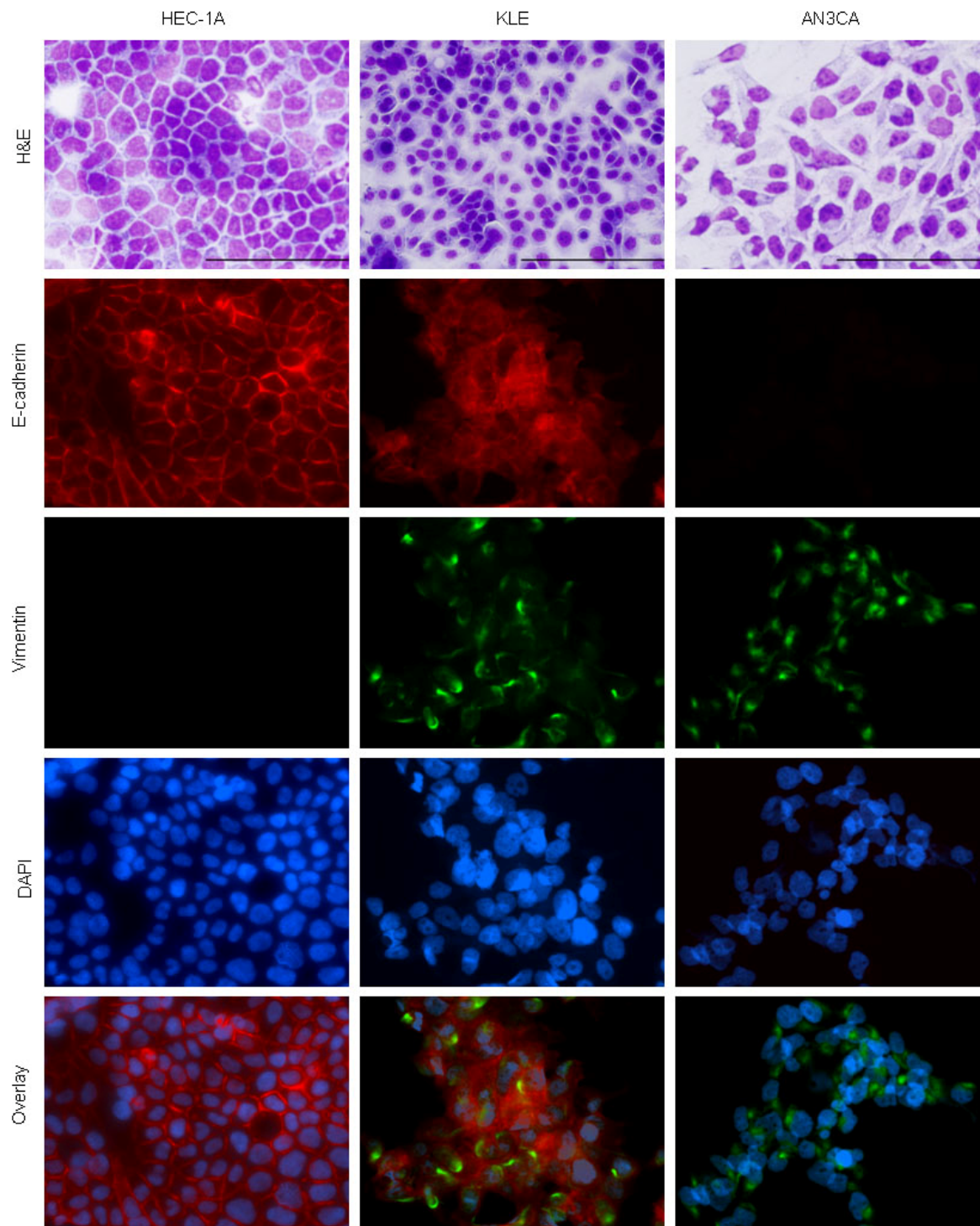


Table 7.2. *CD73* transcripts in endometrial carcinoma cell lines with an epithelial-like, epithelial and mesenchymal-like, or mesenchymal-like phenotype.

Endometrial Carcinoma Cells	CD73 Mean \pm SE	Phenotype
HEC-1B	0.2664 \pm 0.030	Epithelial
HEC-1A	0.1445 \pm 0.009	Epithelial
KLE	0.0328 \pm 0.004	Epithelial-Mesenchymal
ECC-1	0.0057 \pm 0.006	Epithelial-Mesenchymal
Ishikawa	0.0016 \pm 0.000	Epithelial-Mesenchymal
AN3CA	0.0000 \pm 0.000	Mesenchymal

Data are expressed as mean \pm SE molecules of *CD73*/molecules of *18S* ribosomal RNA

Endometrial Carcinoma Cell Origin:

HEC-1B, Grade 2 endometrial endometrioid carcinoma (277)

HEC-1A, Grade 2 endometrial endometrioid carcinoma (277)

KLE, Grade 2, FIGO Stage III endometrial endometrioid carcinoma (228)

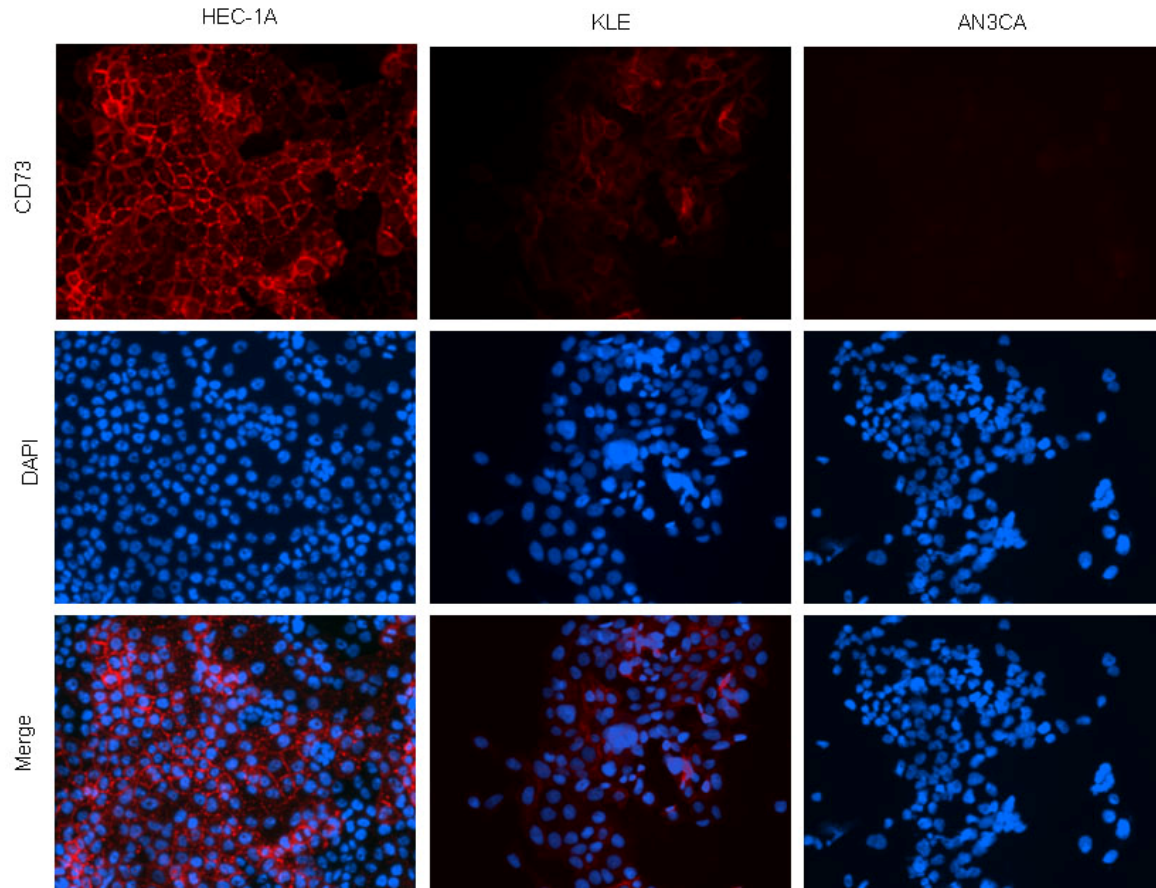
ECC-1, passing of a well-differentiated endometrial carcinoma in nude mice (278)

Ishikawa, Grade 1 endometrial endometrioid carcinoma (279)

AN3CA, lymph node metastasis of an undifferentiated endometrial carcinoma (280, 281)

Figure 7.4. CD73 immunofluorescence in endometrial carcinoma cells with epithelial-like, epithelial and mesenchymal-like, or mesenchymal-like phenotypes. CD73 immunofluorescence in epithelial-like endometrial carcinoma cells, HEC-1A, epithelial and mesenchymal-like endometrial carcinoma cells, KLE, and mesenchymal-like endometrial carcinoma cells, AN3CA. HEC-1A cells express membranous CD73. KLE express CD73 in a few cells. CD73 expression in AN3CA is absent. Image magnification 20X

Figure 7.4



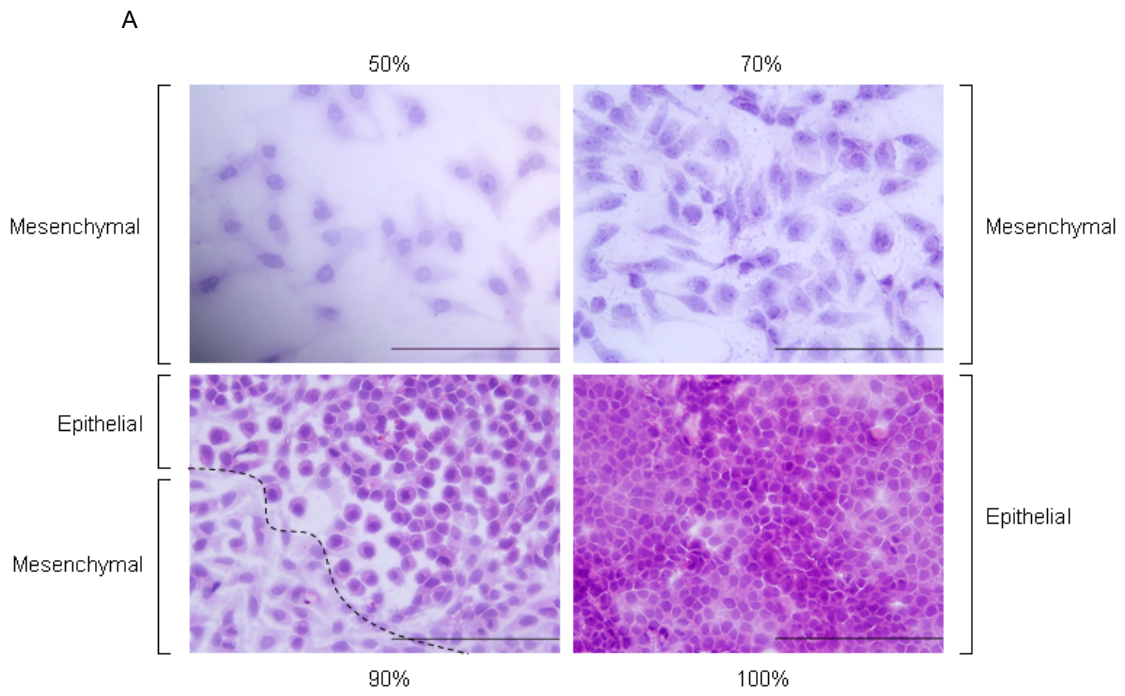
WISH-HeLa, a cervical carcinoma cell sub-line, spontaneously transitions from a mesenchymal-like phenotype to an epithelial-like phenotype with increasing cell confluency

WISH (Wistar Institute Susan Hayflick) is an epithelial cell line originally derived from human amnion epithelium (282, 283). However, later it was discovered to be contaminated with the cervical carcinoma cell line, HeLa (Henrietta Lacks) (284). WISH-HeLa are described to retain characteristics both of amnion epithelium and that of a transformed state (285). With changing density of WISH-HeLa cells (as they become increasingly confluent), the phenotype of the cells transitions from mesenchymal-like (spindle morphology) to epithelial-like (cobblestone morphology) (Figure 6.5 A) This change was not due to cell crowding effects that occur with extreme cell density, as this transformation was clearly seen at 90% confluency. In determining the relative area and perimeter of WISH-HeLa cells at the indicated confluencies, epithelial-like confluencies (90% and 100% confluency) and mesenchymal-like confluencies (50% and 70% confluency) are distinct (Figure 6.5 B). Likewise, cell-cell adhesion expression varies between the mesenchymal-like and epithelial-like confluencies. E-cadherin protein expression was absent at 70% confluence, but was detectable at 100% confluency (Figure 6.5 C). Though collectively WISH-HeLa cells do not undergo a complete transition from a mesenchymal to an epithelial phenotype, as can be seen by cytoplasmic displaced E-cadherin and simultaneous expression of vimentin, a few patches of cells do appear to completely convert phenotypes. This is shown by representative images of WISH-HeLa cells at 2 day post-confluency (Figure 6.5 C). Membrane expressed E-cadherin is observed with simultaneous loss of vimentin. In addition, the transcript levels of tight junction member and mediator of barrier function, *claudin-1*, increases significantly at epithelial-like confluencies (Figure 6.5 D). *A2BR* transcripts remained unchanged with the changing WISH-HeLa cell confluencies (Figure 6.5 E). This finding highlights that increased gene expression (*CD73* and *claudin-1*) seen with increasing WISH-HeLa cell confluencies is not a

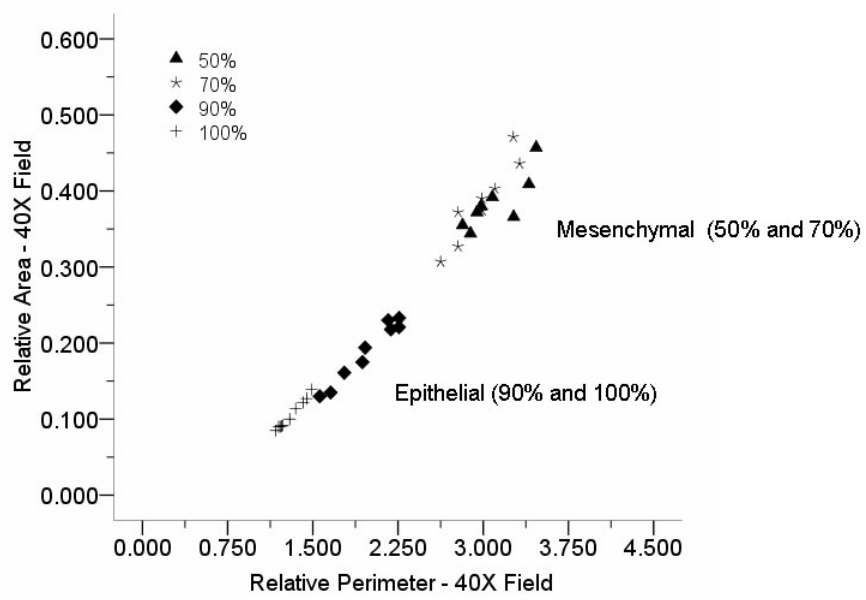
global occurrence. To our knowledge, this is the first study to describe the phenotypic and genotypic changes associated with WISH-HeLa cell confluency.

Figure 7.5. Spontaneously transitioning, mesenchymal-to-epithelial, cervical carcinoma cell sub-line, WISH-HeLa, and cell confluency. (A) Hematoxylin and eosin staining of WISH-HeLa cells at varying cell confluencies. A mesenchymal-like phenotype is seen at 50% and 70% confluencies, as indicated by the spindle morphology of the cells. At 90% confluency the transition of phenotypes is apparent by the appearance of cobblestone morphology in selected cells and retention of the spindle morphology in other cells. Change in phenotypes, mesenchymal-like to epithelial-like, is not the result of cell crowding. (B) Relative perimeter and area of WISH-HeLa cells were measured at indicated confluencies. Mesenchymal-like (50% and 70% confluencies) and epithelial-like (90% and 100% confluencies) confluencies are distinct. (C) E-cadherin immunofluorescence is absent from WISH-HeLa cells at 70% confluency. Cytoplasmic expression of E-cadherin is seen at 100% confluency. A few cells appear to undergo complete mesenchymal-to-epithelial transition at 2 day post-confluency as indicated by membrane expressed E-cadherin (inset) and concurrent loss of vimentin (inset, asterisk). (D) qRT-PCR of WISH-HeLa cell confluencies show transcript levels for tight junction member and barrier function mediator, *claudin-1*, to increase significantly in the confluencies that are most epithelial-like. (E) *A2BR* transcripts remained unchanged among the WISH-HeLa cell confluencies. Error bars represent ± 1 SE. Image magnification 20X

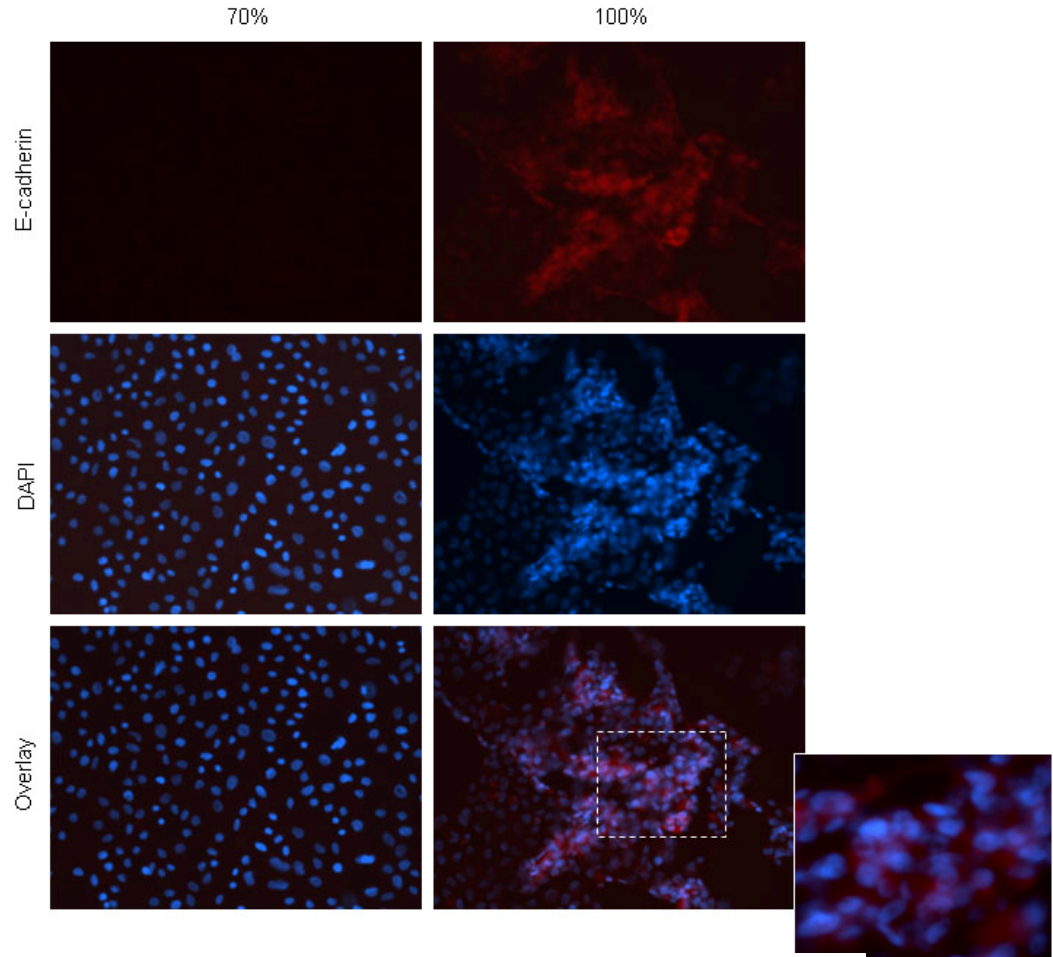
Figure 7.5



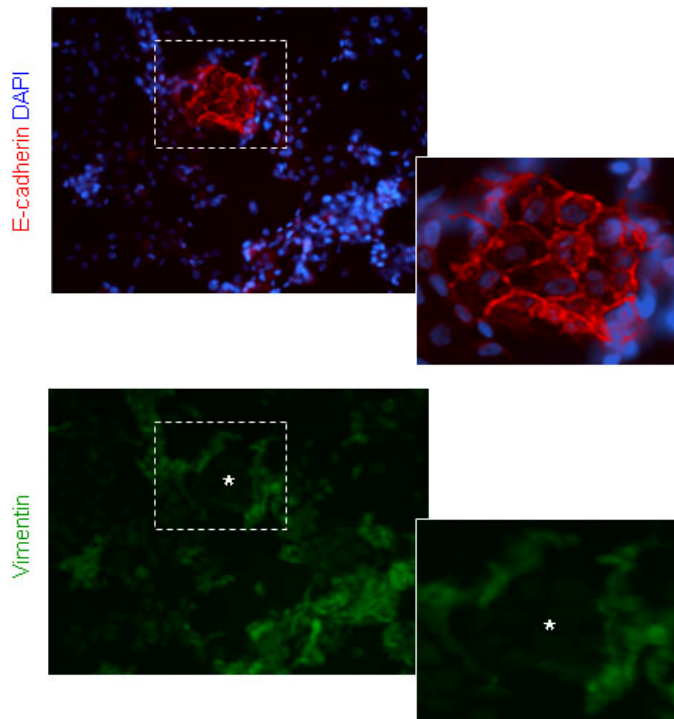
B



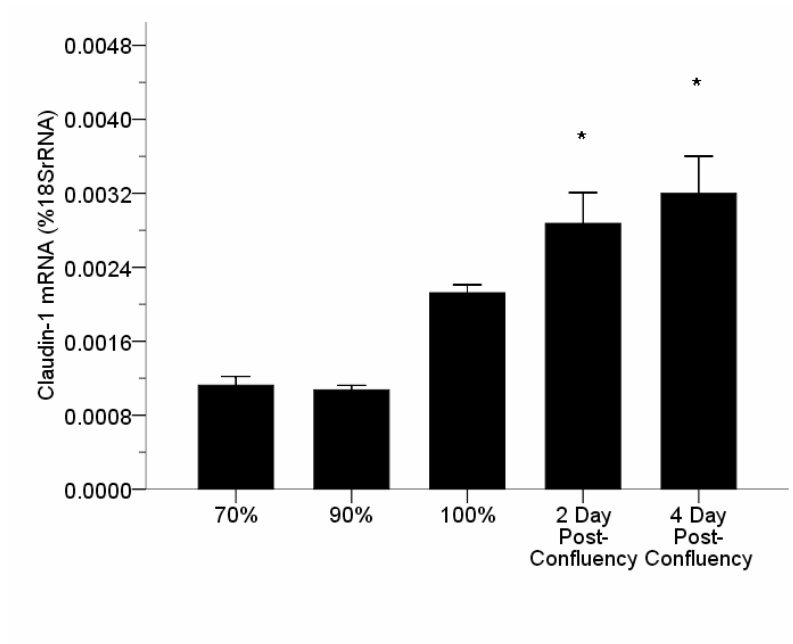
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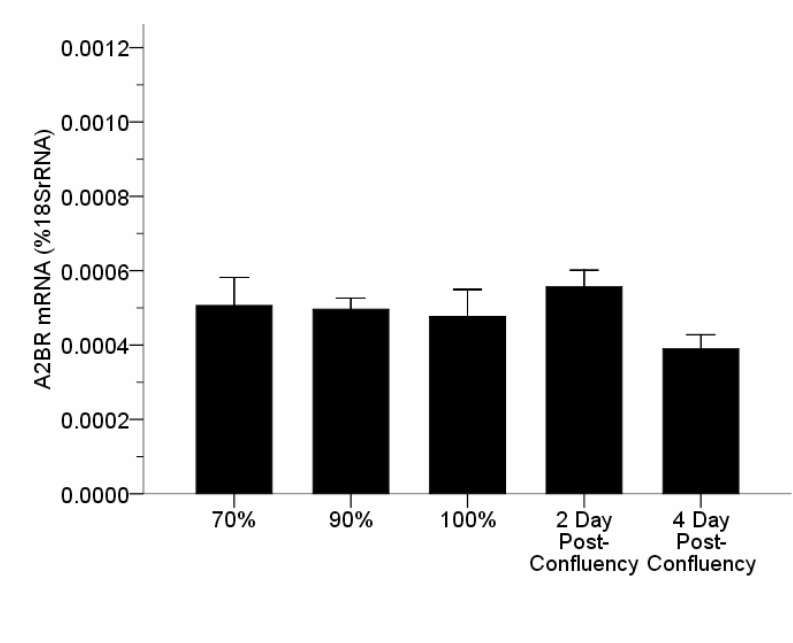
2 Day Post-Confluency



D



E

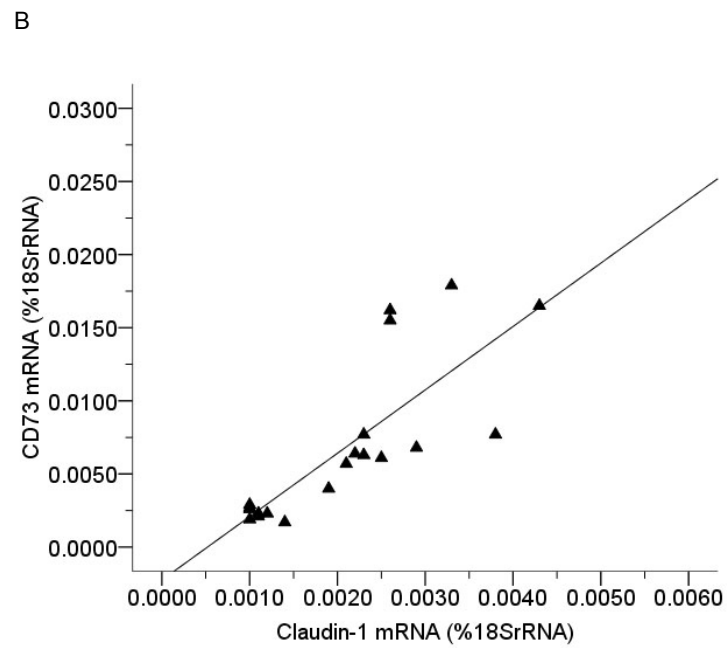
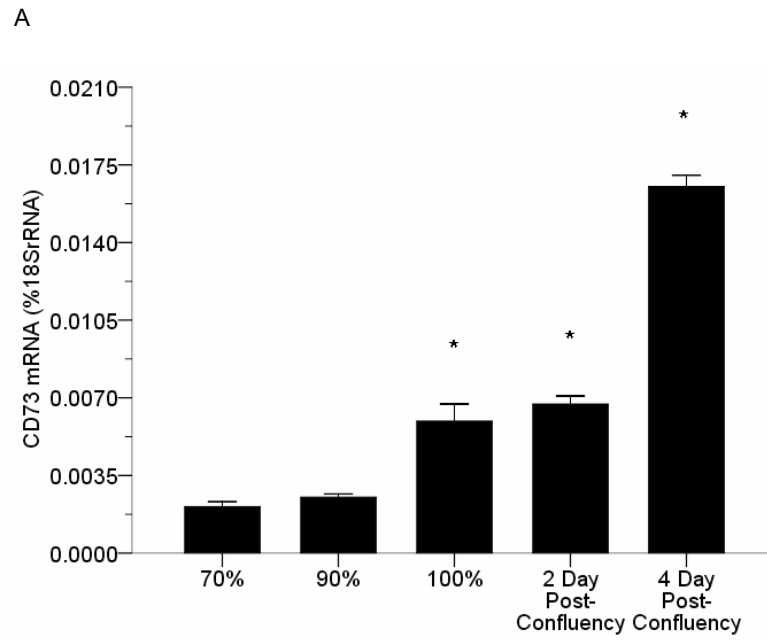


CD73 is highly expressed in the epithelial-like phenotype of WISH-HeLa

The studies of this specific aim thus far have demonstrated an apparent correlation between CD73 expression and the epithelial phenotype of carcinoma cells. To establish this direct association, we took advantage of the mesenchymal and epithelial growth patterns of WISH-HeLa and evaluated CD73. Similar to *claudin-1*, *CD73* transcripts increased significantly at epithelial-like confluencies, 100%, 2 day post-confluency, and 4 day post-confluency (Figure 7.6 A). *CD73* transcripts were highly and positively correlated with those of *claudin-1* transcripts (Figure 7.6 B and Table 7.3). *CD73*'s protein expression was also apparent in the regions of WISH-HeLa cells with the epithelial-like phenotype (90% confluency) (Figure 7.6 C). These studies suggest that epithelial CD73 expression is involved in regulating essential features of the epithelial phenotype, such as cell-cell adhesions.

Figure 7.6. CD73 expression in spontaneously transitioning cervical carcinoma cell sub-line, WISH-HeLa. (A) *CD73* and (B) *A2BR* transcript levels were assessed by qRT-PCR in WISH-HeLa at increasing confluencies. (A) *CD73* transcripts significantly increased at epithelial-like confluencies, 100%, 2 Day Post-Confluency, and 4 Day Post-Confluency. (B) *CD73* protein expression is apparent in the regions of WISH-HeLa cells with the epithelial-like phenotype (90% confluency). 90% confluency image, mesenchymal-like cells are located above the dashed line. Error bars represent ± 1 SE. Image magnification 20X.

Figure 7.6



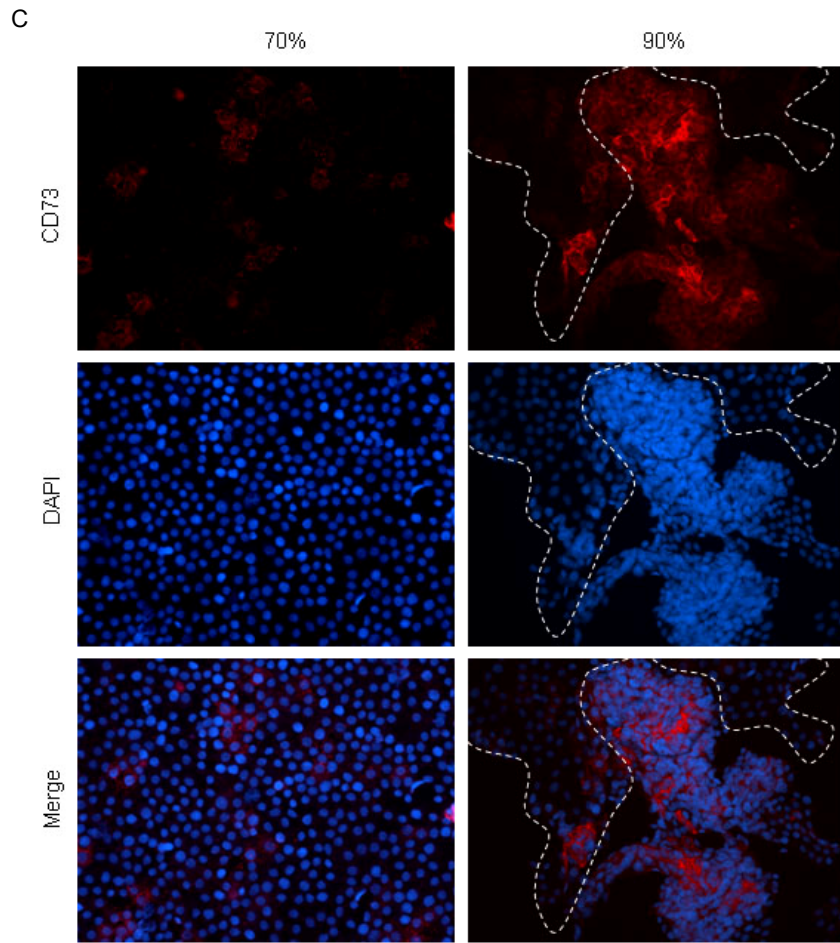


Table 7.3. Correlation coefficient for *CD73* and *Claudin-1* in spontaneously transitioning cervical carcinoma cell sub-line, WISH-HeLa

WISH-HeLa	Pearson's Correlation Coefficient	<i>p</i>
<i>CD73</i> vs <i>Claudin-1</i> Transcripts	0.796	0.00003

Endometrial expressed CD73 is catalytically active and its specific activity is highly correlated to its level of expression

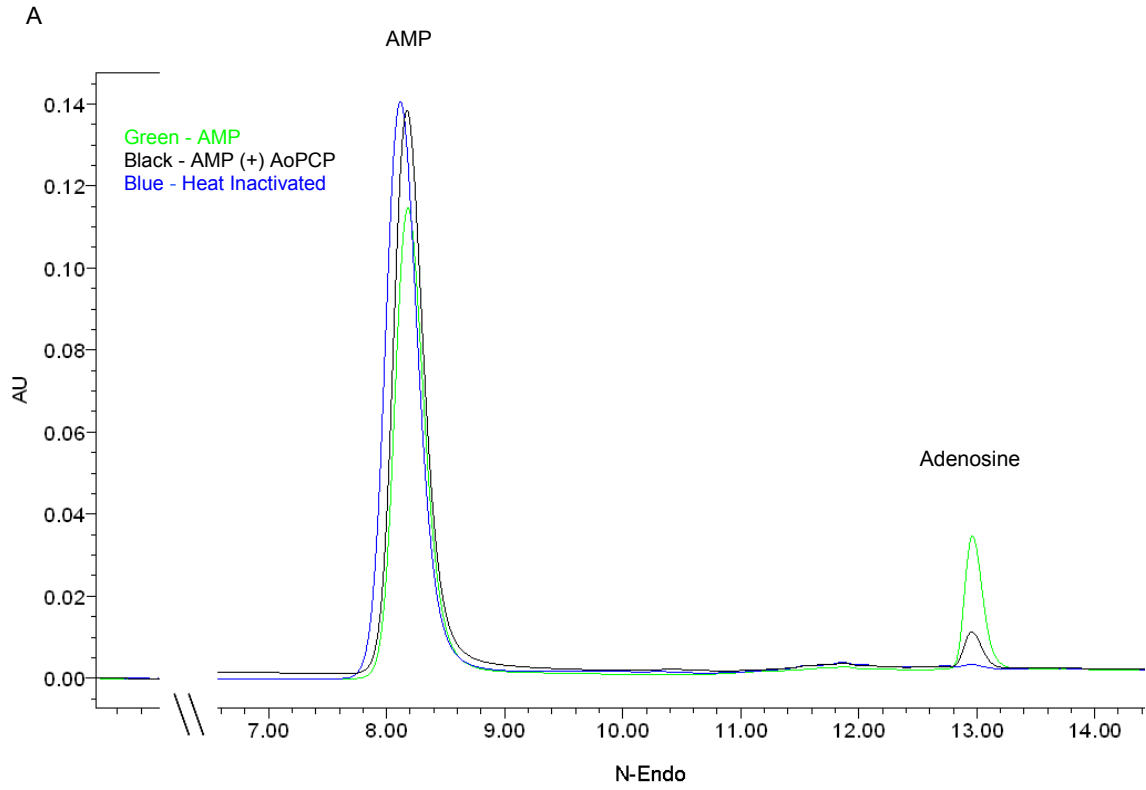
It is generally accepted that CD73's catalytic activity, the production of adenosine, is its primary function and is central to adenosine-mediated barrier function. Non-catalytic activities, including mediating cell-extracellular matrix (ECM) adhesions (163, 164), have only been briefly described. This specific aim has shown thus far that epithelial cells of normal endometrium and carcinoma cells of well-differentiated ECs maintain relatively similar levels (high intensity) of CD73 expression. This suggests that CD73-generated adenosine and adenosine's barrier function may be functioning to inhibit the progression of these well-differentiated ECs to more poorly-differentiated and invasive ECs that are more aggressive (metastatic in nature). The primary mechanism in which CD73 contributes to epithelial barrier function is through the catalytic production of adenosine from the phosphohydrolysis of AMP. Studies in this dissertation thus far have demonstrated that CD73-generated adenosine induces barrier function in epithelial cells of the endometrium (*in vivo* model) and that CD73 is primarily expressed in epithelial cells of the normal endometrium. Our data suggest that CD73 plays a major role in the generation of adenosine and that adenosine mediates increased barrier function, which functions to inhibit EC progression, so it is relevant to demonstrate that normal endometrium and EC expressed CD73 is catalytically active. To determine this, the specific activity of CD73 was assessed in normal endometrium, well- and poorly-differentiated ECs, and endometrial carcinoma cells, HEC-1A, KLE, and AN3CA by reverse phase high performance liquid chromatography (RP-HPLC). CD73 specific activity was determined by incubating samples with AMP or AMP and CD73 catalytic inhibitor, α , β -methylene diphosphate (AoPCP), or by heat inactivation of CD73. A representative chromatogram (Figure 7.7 A) shows the retention times and peaks for AMP and adenosine which are typical of a nucleotidase (CD73) assay. Similar to the patterns of qRT-PCR and immunofluorescent expression for CD73, CD73's specific activity was the highest in normal

endometrium (N-Endo) and Grade 1 endometrioid endometrial carcinomas (G1 EEC) and significantly lower in Grade 3 endometrioid endometrial carcinomas (G3 EEC) (Figure 7.7 C and Table 7.4). A representative modified chromatogram demonstrates the adenosine peaks of a single sample each of N-Endo, G1 EEC, and G3 EEC samples following incubations of AMP (green) AMP and AoPCP (black) or heat inactivation (blue) (Figure 7.7 B). Importantly, the data clearly show that expressed CD73 in N-Endo, G1 EEC, and G3 EEC can function catalytically to produce adenosine. Amongst endometrial carcinoma cells, representative of epithelial-like (HEC-1A), epithelial and mesenchymal-like (KLE), and mesenchymal-like (AN3CA), the catalytic activity of CD73 was associated with levels of protein expression (Figure 7.8 and Table 7.5). For ECs and endometrial carcinoma cells, a significant correlation was observed between *CD73* transcript levels and CD73 enzymatic activity (Figure 7.9 and Table 7.6).

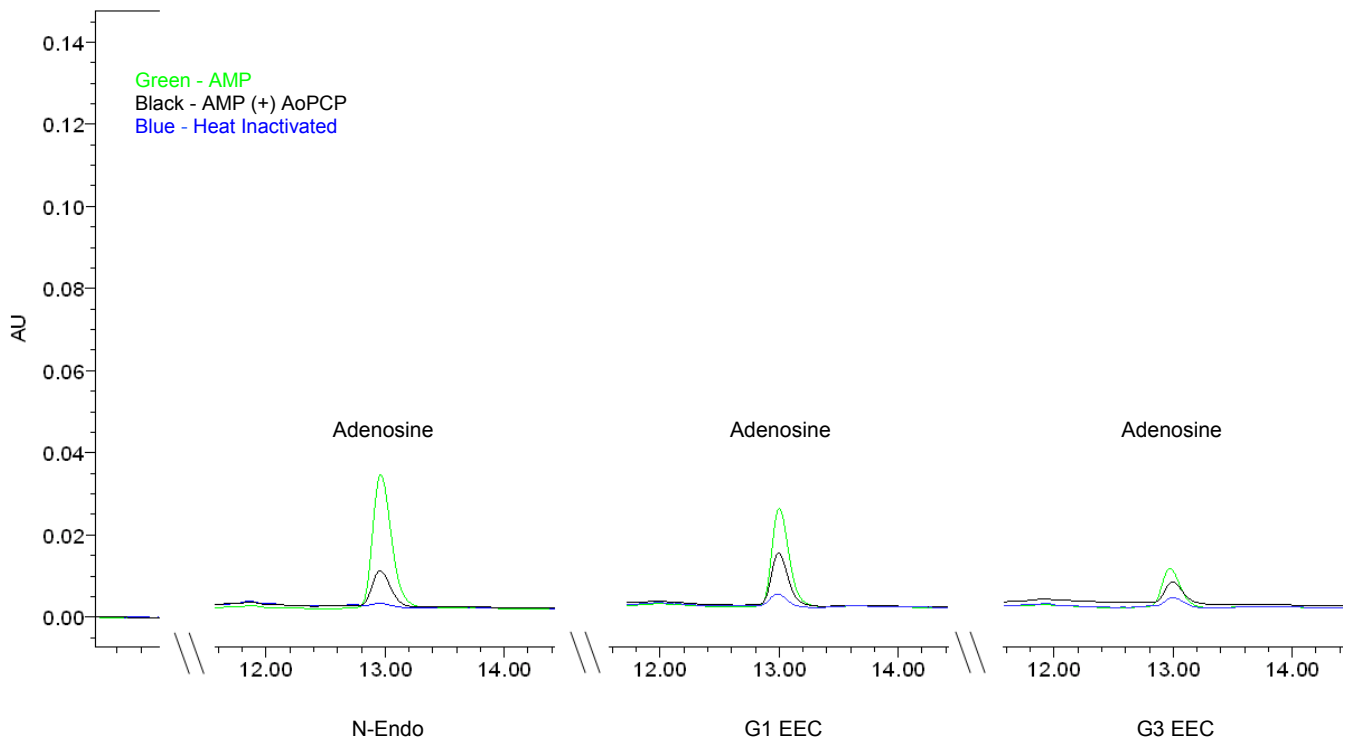
Figure 7.7. CD73 specific activity in normal endometrium and endometrial carcinomas.

CD73 specific activity was determined by reverse phase high performance liquid chromatography (RP-HPLC) in normal endometrium and endometrial carcinomas, G1 EEC and G3 EEC. Adenosine production was measured at an absorbance of 260nm. (A) Representative chromatogram with peaks typical of a nucleotidase assay. Retention times for AMP and adenosine are 8 minutes and 13 minutes, respectively. CD73 specific activity was determined by using heat inactivation (blue line, chromatogram) and a catalytic inhibitor, α,β -methylene diphosphate (AoPCP) (black line, chromatogram) (B) Modified chromatograms representing adenosine peaks of a representative sample of N-Endo, G1 EEC, and G3 EEC following incubation with AMP, AMP and AoPCP, or undergoing heat inactivation. CD73 specific activity was calculated by subtracting heat inactivated and AMP and AoPCP values from AMP samples. Y axis is relative. An adenosine standard was run prior to this study and used to obtain plotted values shown in (C). (C) Data were plotted using box and whisker plots (bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median)). Specific activity for CD73 was significantly lower in G3 EECs. Error bars represent ± 1 SE. (* $p < 0.05$)

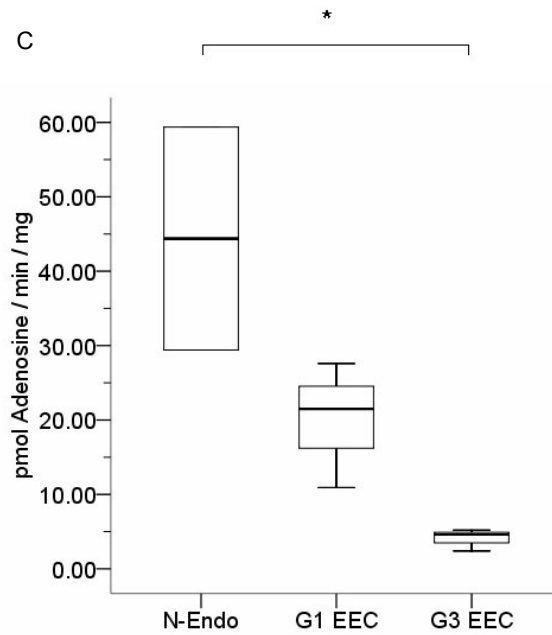
Figure 7.7



B



C



**Table 7.4. CD73 specific activity in normal endometrium and endometrial carcinomas –
Data summary from Figure 7.7 C**

	N	aMean ± SE	p value (vs N-Endo)
N-Endo	2	44.38 ± 21.18	--
G1 EEC	3	20.01 ± 8.44	NS
G3 EEC	3	4.07 ± 1.47	0.02

^aData are expressed as mean pmol Adenosine / min / mg

Abbreviations:

N-Endo, histologically normal endometrium

G1 EEC, Grade 1 endometrioid endometrial carcinoma; well-differentiated

G3 EEC, Grade 3 endometrioid endometrial carcinoma; poorly-differentiated

Figure 7.8. CD73 specific activity in endometrial carcinoma cells, HEC-1A, KLE, and AN3CA. CD73 specific activity was determined by reverse phase high performance liquid chromatography (RP-HPLC) in endometrial carcinoma cells with an epithelial-like (HEC-1A), epithelial and mesenchymal-like (KLE), and mesenchymal-like (AN3CA) phenotype. (A) Data were plotted using a box and whisker plots: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent. Specific activity for CD73 was significantly lower in KLE and AN3CA cells compared to HEC-1A. Error bars represent ± 1 SE. (* $p < 0.05$)

Figure 7.8

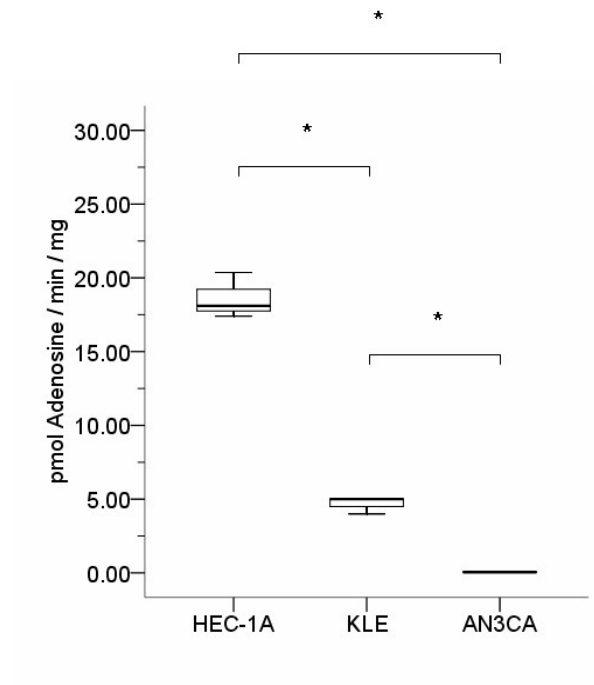


Table 7.5. CD73 specific activity in endometrial carcinoma cells, HEC-1A, KLE, and AN3CA – Data summary from Figure 7.8

	N	aMean ± SE	p value (vs HEC-1A)
HEC-1A	3	18.62 ± 0.89	--
KLE	3	4.67 ± 0.33	0.000005
AN3CA	3	0.06 ± 0.01	0.000001

^aData are expressed as mean pmol Adenosine / min / mg

Phenotype:

HEC-1A, epithelial-like

KLE, epithelial-mesenchymal-like

AN3CA, mesenchymal-like

Figure 7.9. CD73 transcripts and specific activity correlations in normal endometrium, endometrial carcinomas, and endometrial carcinoma cells. CD73 transcript levels and CD73 specific activity were simultaneously graphed and assessed for linear (R^2) values. (A) CD73 transcript levels and CD73 specific activity of normal endometrium (N-Endo) and well- and poorly-differentiated ECs, Grade 1 and 3 endometrioid endometrial carcinoma (G1 EEC and G3 EEC). Linear values show a high correlation, Pearson's correlation coefficient, 0.968; $p=0.00008$. (B) CD73 transcript levels and CD73 specific activity of epithelial-like endometrial carcinoma cells, HEC-1A, epithelial-mesenchymal-like endometrial carcinoma cells, KLE, and mesenchymal-like endometrial carcinoma cells, AN3CA. Linear values show a high correlation, Pearson's correlation coefficient, 0.991; $p=0.0000002$.

Figure 7.9

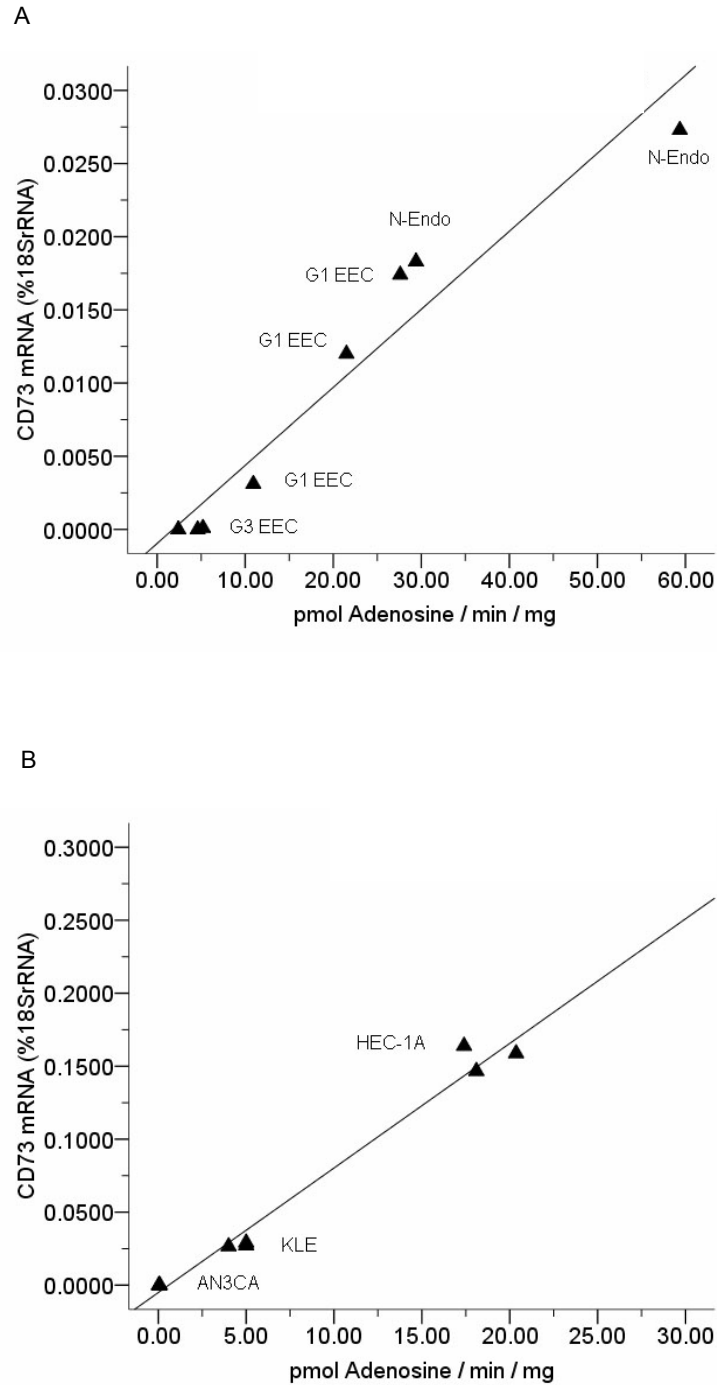


Table 7.6. *CD73* transcripts and specific activity correlations in normal endometrium, endometrial carcinomas, and endometrial carcinoma cells – Data summary from Figure 7.9

	Pearson's Correlation Coefficient	<i>p</i>
N-Endo, G1 EEC, G3 EEC	0.968	0.00008
HEC-1A, KLE, AN3CA	0.991	0.0000002

Abbreviations:

N-Endo, histologically normal endometrium

G1 EEC, Grade 1 endometrioid endometrial carcinoma; well-differentiated

G3 EEC, Grade 3 endometrioid endometrial carcinoma; poorly-differentiated

Phenotype:

HEC-1A, epithelial-like

KLE, epithelial-mesenchymal-like

AN3CA, mesenchymal-like

CD73's catalytic activity is localized primarily to normal epithelial and well-differentiated carcinoma cells, however it is lost in carcinoma cells of poorly-differentiated histotypes

To determine the localization of CD73's catalytic activity the normal endometrium and endometrial carcinomas, a lead sulfide method was used to observed the precipitated byproduct (hydrolyzed phosphate) of CD73's phosphohydrolysis of AMP (231). A detailed schematic of this enzyme histochemistry method is shown in Figure 7.10. Similar to the protein expression pattern seen for CD73 in normal endometrium, glandular epithelium exhibited the most apparent catalytic activity for CD73 (Figure 7.11). Strong catalytic activity was also found in carcinoma cells of Grade 1 endometrioid endometrial carcinomas (G1 EEC), which is similar to that seen for its protein expression. CD73 catalytic activity in Grade 3 endometrioid endometrial carcinomas (G3 EEC) and uterine papillary serous carcinomas (UPSC) was lower than that of N-Endo or G1 EEC. Most of the catalytic activity for CD73 in G3 EEC and UPSC is found in the surrounding stroma/connective tissue. As was expected, endometrial carcinoma cells showed CD73 catalytic activity (relatively high or low) that was comparative to CD73's protein expression seen within these cells (Figure 7.12). Higher CD73 catalytic activity was found in epithelial-like endometrial carcinoma cells (HEC-1A and HEC-1B), as compared to endometrial carcinoma cells that were epithelial-mesenchymal-like (KLE) or mesenchymal-like (AN3CA). Importantly, CD73 activity is greatly apparent at the membrane of the epithelial-like (well-differentiated) carcinoma cells. These data taken together support that normal epithelial cell or well-differentiated carcinoma cell-expressed CD73 functions to produce adenosine, which mostly likely induces barrier function. In well-differentiated ECs this may be a feature that restricts the migration and invasiveness of the carcinoma cells. The following specific aim (Specific Aim 4) addresses this potential.

Figure 7.10. Enzyme histochemistry reaction for CD73. Tissue or carcinoma cell expressed CD73's phosphohydrolysis of AMP can be visibly viewed and cellular localization determined by precipitation of the hydrolyzed phosphate from AMP by a lead sulfide method. This technique was modified from Wachstein, M. and Meisel, E., Histochemistry of hepatic phosphatase at a physiological pH with special reference to the demonstration of bile canaliculi, Am. J. Clin. Pathol. 1957;27:13-23 (231).

Figure 7.10

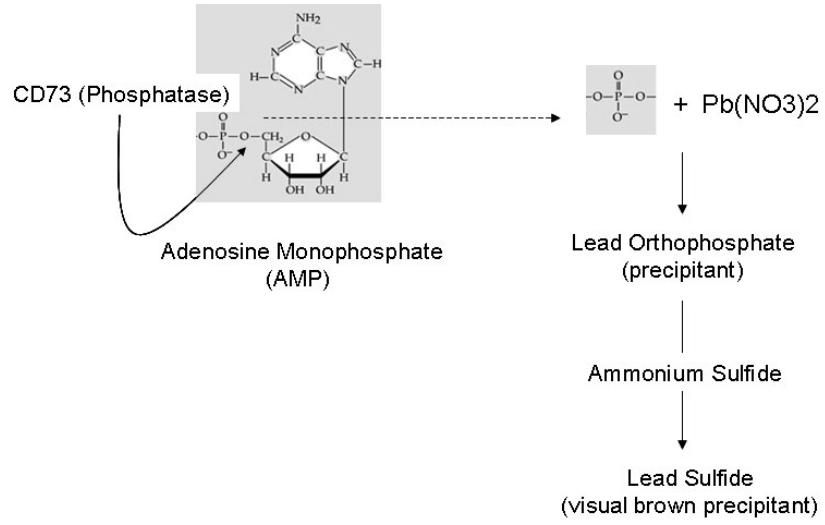


Figure 7.11. CD73 enzyme histochemistry in normal endometrium, well-differentiated and poorly-differentiated endometrial carcinomas. (A) Cryosections of normal endometrium and endometrial carcinomas were microscopically examined for localization of CD73's catalytic activity using enzyme histochemistry. Catalytic activity of CD73 is highest in glandular epithelium of normal endometrium (N-Endo). Relatively high catalytic activity also occurs in G1 EEC, with lower activity in G3 EEC and UPSC. Much of the CD73 activity seen in G3 EEC and UPSC is present in surrounding stromal cells/connective tissue. Image magnification 20X. Arrows indicate glandular epithelial cells (N-Endo) or carcinoma cells (G1 EEC, G3 EEC, UPSC)

Figure 7.11

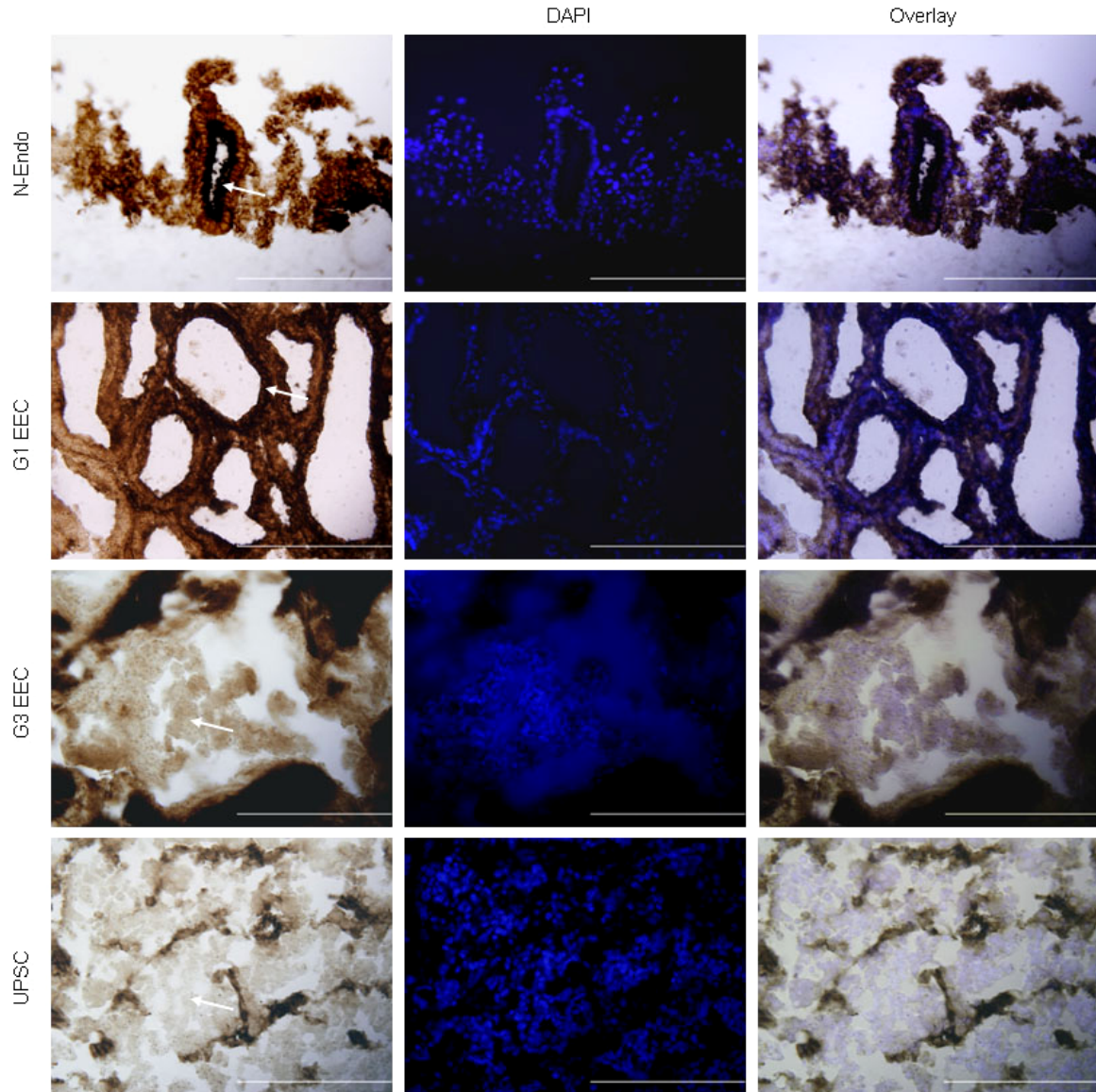
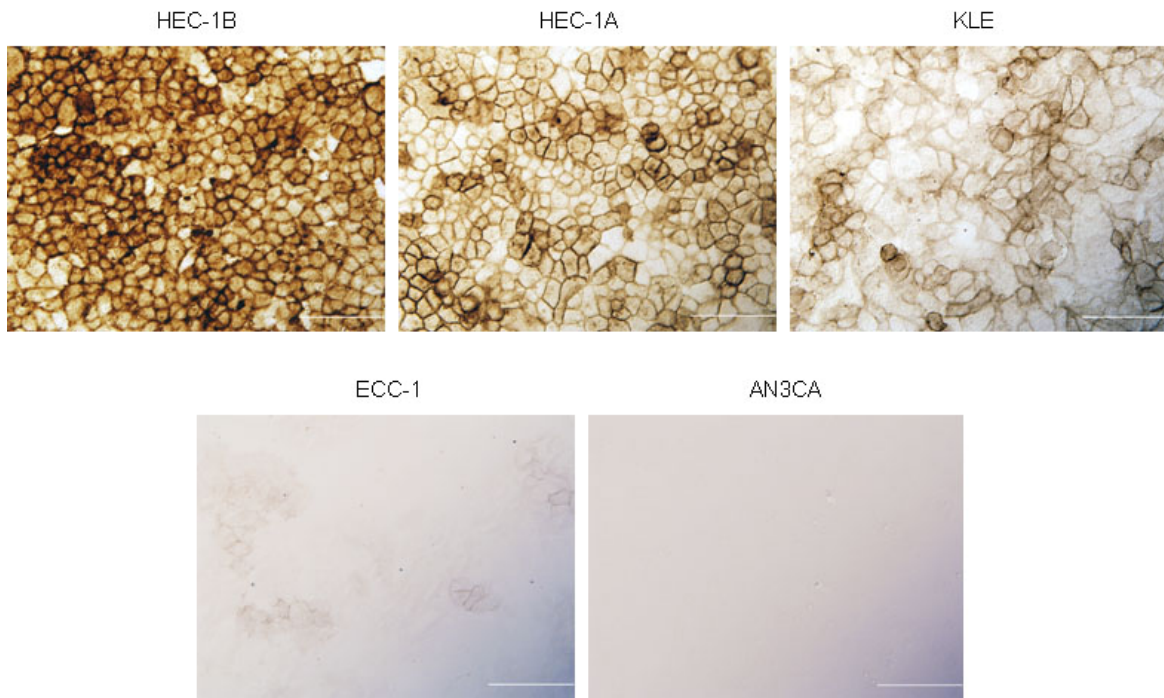


Figure 7.12. CD73 enzyme histochemistry in endometrial carcinoma cells. CD73's catalytic activity was assessed in endometrial carcinoma cells with an epithelial-like (HEC-1B and HEC-1A), epithelial-mesenchymal-like (KLE and ECC-1), and mesenchymal-like (AN3CA) phenotype. (A) CD73 catalytic activity is higher in HEC-1B and HEC-1A, than in KLE. CD73's catalytic activity is primarily localized to the membrane in these cells. (B) CD73 catalytic activity in epithelial-mesenchymal-like (ECC-1) and mesenchymal-like (AN3CA) endometrial carcinoma cell lines. CD73 catalytic activity is low to absent in ECC-1 and AN3CA cells. Image magnification 10X.

Figure 7.12



Summary

Characterizing the expression pattern of CD73 in resident cells of the normal endometrium and endometrial carcinomas is important to establishing the significance of CD73 down-regulation in EC. Specific aim 1 studies have shown that CD73 is down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs compared to normal endometrium and well-differentiated ECs. Data from this specific aim has established that the primary expression of CD73 is within epithelial cells of the normal endometrium. This high expression seen in epithelial cells of the normal endometrium was similar to levels seen among carcinoma cells of well-differentiated ECs. Published studies have demonstrated that well-differentiated ECs typically retain expression of cell-cell adhesion proteins, such as E-cadherin, and are generally non-invasive ECs. Most importantly, data shown herein demonstrate that the loss of CD73 expression in poorly-differentiated histotypes occurs within the carcinoma cells. *In vitro* data has clearly established that CD73 expression is related and highly correlated to an epithelial phenotype of endometrial and cervical carcinoma cells. Epithelial- and carcinoma-expressed CD73 appears to be a caretaker of the epithelial phenotype. This caretaker responsibility is likely related to the impact CD73-generated adenosine elicits upon cell-cell adhesions and their individual proteins. The generation of adenosine by CD73 is the primary feature of adenosine-mediated barrier function. Results obtained using RP-HPLC and enzyme histochemistry have confirmed that CD73 expressed in normal endometrium and ECs is catalytically active.

CHAPTER EIGHT

Results

Specific Aim 4. Show that CD73-generated adenosine and adenosine's activation of A2BR inhibit the migration and invasion of endometrial carcinoma cells.

Introduction

Of the many effects the loss of cell-cell adhesions have on carcinoma cells, the increased potential for carcinoma cells to migrate and invasive may be among the most concerning. Of all cancer related mortalities, 90% are related to the dissemination of cancer cells. The process of carcinoma cell migration and invasion is understood as the movement of individual cancer cells or groups of cancer cells from the primary site of origin to entering into lymphatic vessels or the bloodstream, ultimately seeding in a distant tissue or organ (286). Carcinoma cell migration and invasion are regulated by a broad spectrum of activated proteins and motility-impacting events including the expression and outside-in signaling of integrins, activation of matrix-degrading proteinases, such as members of the matrix metalloproteinase family, (MMPs), cell-cell communication, and loss of cell-cell adhesions (reviewed, (287)). The loss of cell-cell adhesions in carcinoma cells has a great impact on the expression and activity of many of these migration and invasion-promoting events and proteins. As for example, loss of cell-cell adhesion proteins cause polarity proteins to be displaced within the cell, which are freely recruited by TGF- β , PI3K, and Wnt signaling to establish front-rear polarization, an event important for cell migration (reviewed, (75)). Loss of the cell-cell adhesion protein, E-cadherin, and its adhesion dependent suppression of EGFR results in the up-regulation of integrins, including $\alpha_5\beta_1$ -integrin, via the EGFR/focal adhesion kinase (FAK) MAPK-dependent signaling pathway (288) and expression of matrix metalloproteinases, including MMP-9 (289-291), which are proteins important in carcinoma cell migration (focal adhesion formation) and disruption of the surrounding

extracellular matrix. The loss of cell-cell adhesions therefore contributes significantly to creating the ideal molecular and environmental conditions needed for carcinoma cells migration and invasion.

Specific aim 1 and specific aim 2 demonstrated that CD73-generated adenosine is important to maintaining endometrial barrier function (specific aim 2) and that CD73 is significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs (specific aim 1). Importantly, specific aim 3 demonstrated that epithelial cell/carcinoma cell expressed CD73 is catalytically active, and that such catalytically active CD73 is preferentially expressed in epithelial cells compared to adjacent stromal cells. These data support the theory that CD73-generated adenosine and adenosine's activation of A2BR function to inhibit the progression of ECs, potentially by influencing cell-cell adhesions and their individual proteins. An interesting point to consider is that adenosine-mediated induction of barrier function occurs most generally as a response to cellular stress. Cellular stressors are common among all cancers. Carcinoma cells are known to constantly experience hypoxia regardless of stage of disease. Likewise, hypoxia is a known promoter of molecular and cellular events that promote aggressiveness and invasiveness of carcinoma cells. This is particularly true for EC (269, 270, 292, 293). Maintaining barrier function in hypoxic stressed carcinoma cells is counteractive to the hypoxia-induced progression of EC. Therefore, we hypothesized that hypoxia-induced tissue protective events, such as CD73-generated adenosine's barrier function, which in normal tissues are necessary to maintain homeostasis, would need to be down-regulated in EC for the carcinoma cells to progress (migration and invasion). This specific aim examines the potential of CD73, CD73's generation of adenosine, and adenosine's activation of A2BR to inhibit the migration and invasion of well-differentiated EC cells. To accomplish this specific aim, the established *in vitro* modified and unmodified two-chamber migration and invasion assay system was used (233).

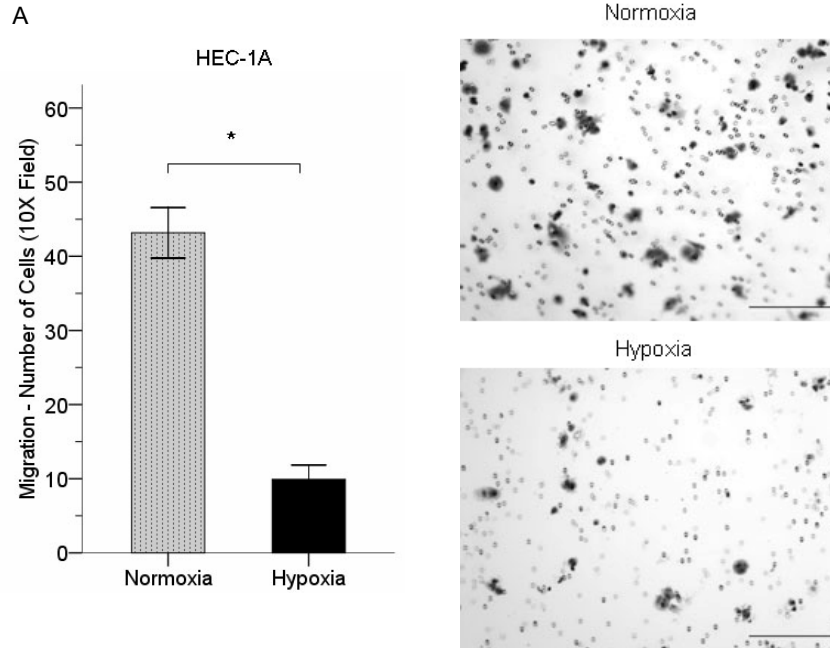
These are well-documented *in vitro* assays for quantitating the migratory or invasive potential of tumor cells.

Hypoxia significantly inhibits the migration and invasion of endometrial carcinoma cells, HEC-1A and HEC-1B

Carcinoma cells are consistently subjected to varying levels of hypoxia *in vivo*. Such hypoxic conditions contrast greatly with the culture conditions routinely used for carcinoma cell experiments *in vitro*. Hypoxia impacts various aspects of carcinoma cell progression and metastasis, including immortalization, transformation, genetic instability, angiogenesis, invasion, metastasis, and therapy resistance. In EC, the relevance of hypoxia in both well-differentiated (Grade 1 endometrioid endometrial carcinomas) and poorly-differentiated (Grade 3 endometrioid endometrial carcinomas) ECs has been established (269, 270, 292, 293). Since CD73-generated adenosine and adenosine's barrier function effect is related to hypoxic stress, the relative migration and invasion of CD73 expressing endometrial carcinoma cells, HEC-1A and HEC-1B, were assessed under normoxic and hypoxic (1% O₂, 5% CO₂) conditions. Recall that in specific aim 2, Figure 6.7., we showed that hypoxia increases steady-state levels of HIF-1 α in these cells. Hypoxic conditions significantly inhibited the migration of HEC-1A cells (Figure 8.1 A and Table 8.1) and migration and invasion of HEC-1B cells (Figure 8.1 A-B and Table 8.1). HEC-1A cells were not studied for invasion due to the too few numbers of cells that would invade to accurately quantify. As shown by Annexin V studies in Figure 8.2 and Table 8.2, the decrease in HEC-1A (A) migration and HEC-1B (B) migration and invasion was not attributed to increased cell death from hypoxia. No statistical differences in cell death between normoxic and hypoxic conditions were observed for either migration (24 hours) or invasion (36 hours) endpoints. These data suggest the potential of CD73's generation of adenosine to be inhibiting EC cell migration and invasion.

Figure 8.1. HEC-1A migration and HEC-1B migration and invasion in normoxic and hypoxic conditions. CD73 expressing endometrial carcinoma cells, HEC-1A (A) and HEC-1B (B,C), were placed in normoxic or hypoxic (1% O₂, 5% CO₂) conditions along with 50μM of 5'AMP. Hypoxia significantly inhibited HEC-1A migration and HEC-1B migration and invasion. Representative photomicrographs from each experiment are shown alongside graphs. Error bars represent ± 1 SE. Image magnification 10X. (*p<0.05)

Figure 8.1



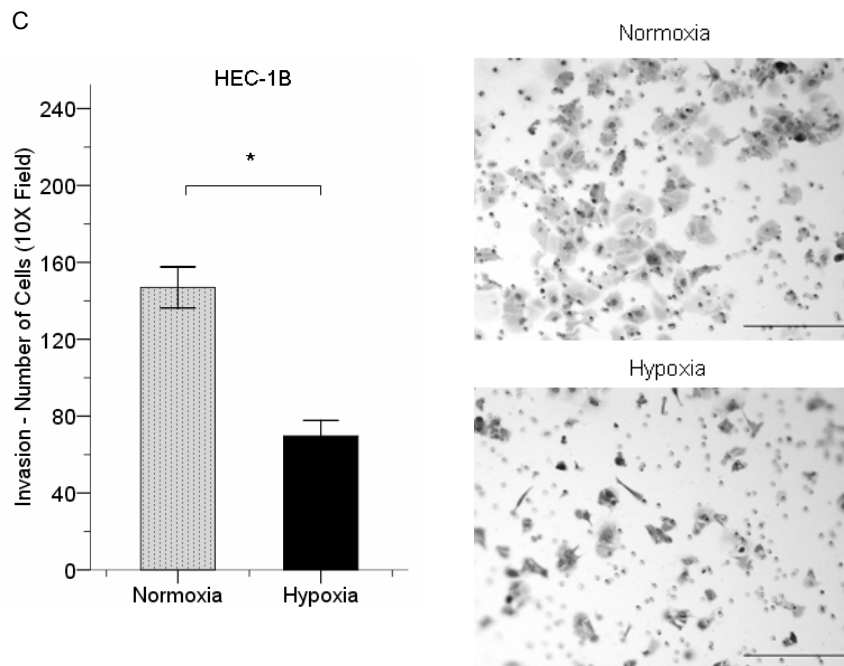
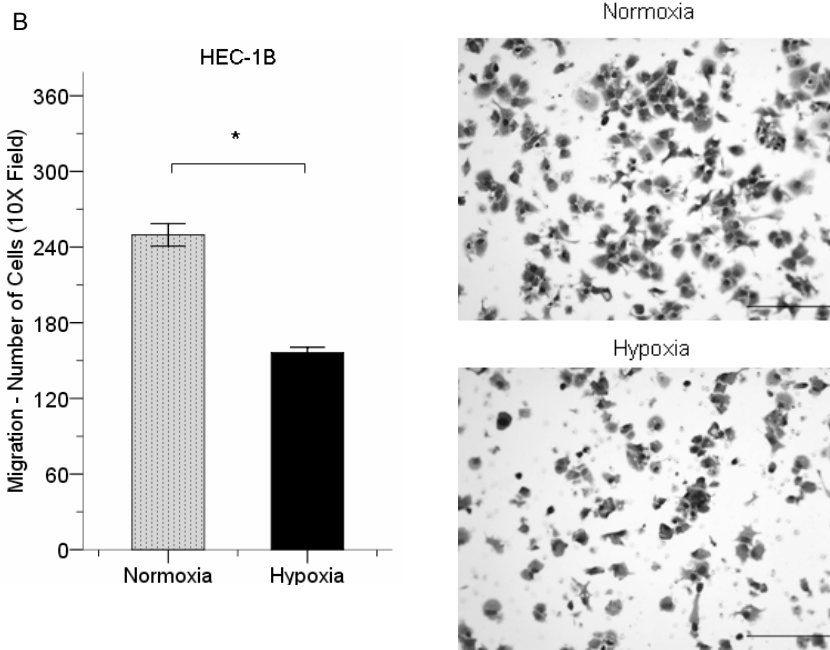


Table 8.1. HEC-1A migration and HEC-1B migration and invasion in normoxic and hypoxic conditions – Data summary from Figure 8.1

	^b N	^a Mean \pm SE	<i>p</i> value (vs normoxia)
HEC-1A - Migration			
Normoxia	3	43.17 \pm 3.41	--
Hypoxia	3	9.92 \pm 1.92	0.00000002
HEC-1B - Migration			
Normoxia	3	249.67 \pm 8.91	--
Hypoxia	3	156.33 \pm 4.25	0.00000000002
HEC-1B - Invasion			
Normoxia	3	146.93 \pm 10.68	--
Hypoxia	3	69.73 \pm 8.07	0.00000005

^aData are expressed as mean cell number of migrated or invaded cells in a 10X field \pm SE

^bN represents the number of migration or invasion inserts per condition

Figure 8.2. Representative histograms of Annexin V at migration and invasion endpoints in normoxic and hypoxic endometrial carcinoma cells, HEC-1A and HEC-1B. Cell death was determined by Annexin V flow cytometry at migration and invasion endpoints in normoxic and hypoxic (1% O₂, 5% CO₂) endometrial carcinoma cells, HEC-1A (A) and HEC-1B (B). Endpoints for migration and invasion were 24 and 36 hours, respectively. No significant differences in cell death between normoxia and hypoxia for HEC-1A or HEC-1B were detected. Data are summarized from triplicate experiments in Table 8.2.

Figure 8.2 A

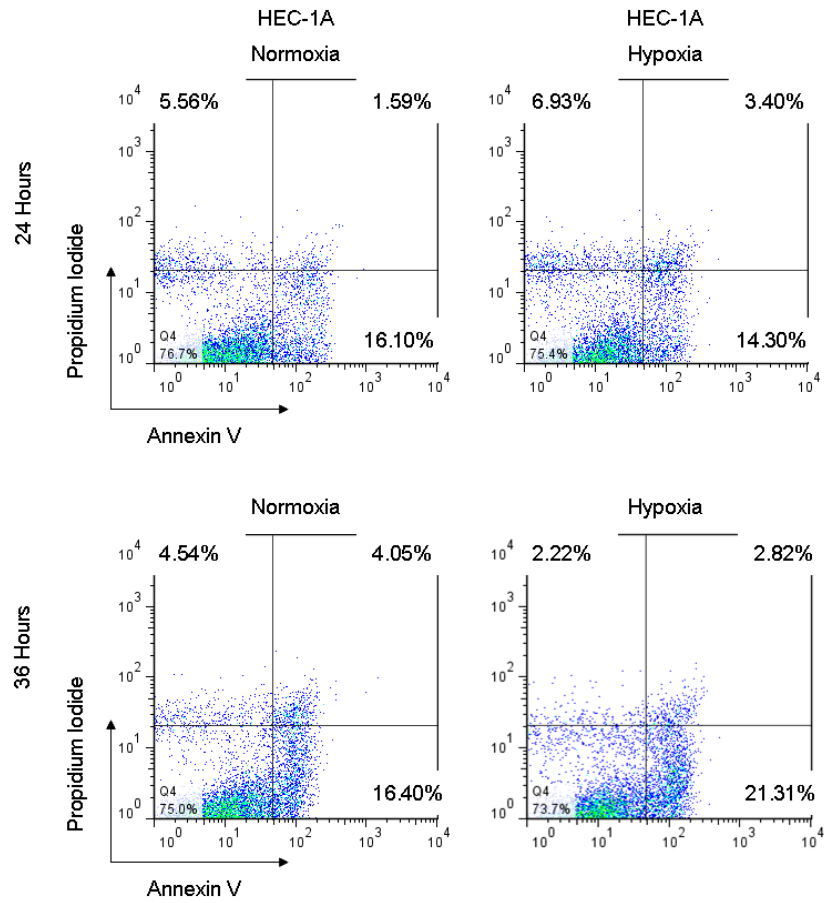


Figure 8.2 B

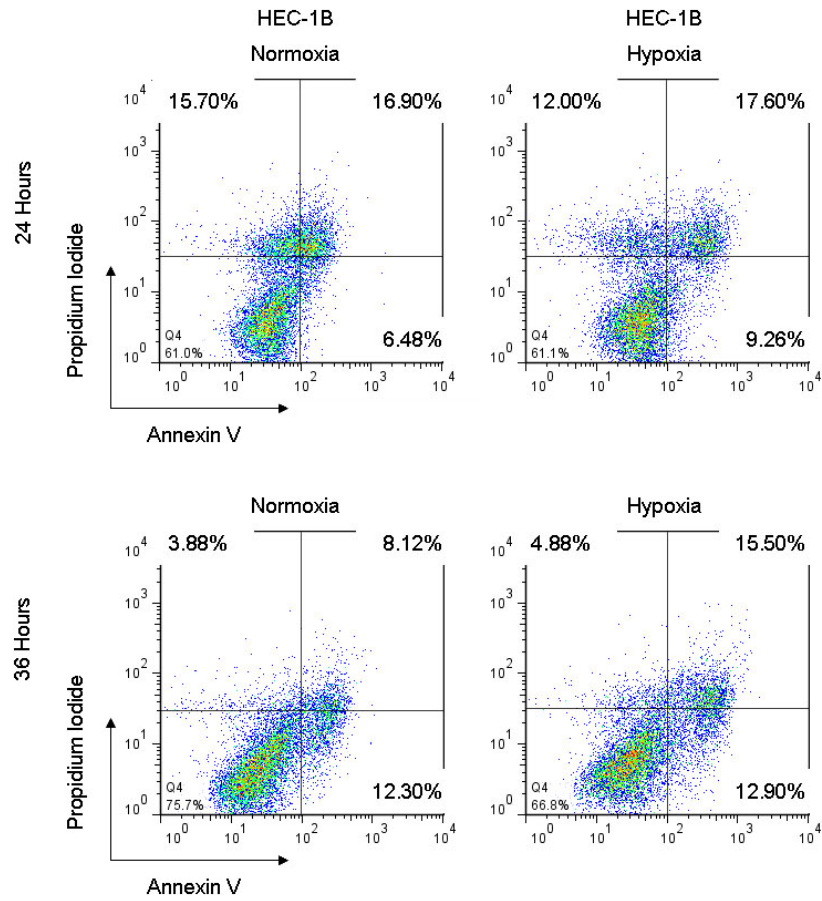


Table 8.2. Representative histograms of Annexin V studies at migration and invasion endpoints in normoxic and hypoxic endometrial carcinoma cells, HEC-1A and HEC-1B – Data summary from Figure 8.2

		Normoxia	Hypoxia	<i>p</i> value (vs normoxia)
	^b N	^a Mean ± SE	^a Mean ± SE	
HEC-1A - 24 Hours				
Live Cells	3	71.90 ± 0.87	71.37 ± 0.70	NS
Dead Cells	3	28.16 ± 1.80	28.67 ± 1.62	NS
HEC-1A - 36 Hours				
Live Cells	3	75.07 ± 2.08	72.60 ± 3.06	NS
Dead Cells	3	24.96 ± 2.22	27.41 ± 3.73	NS
HEC-1B - 24 Hours				
Live Cells	3	65.07 ± 5.26	63.11 ± 5.26	NS
Dead Cells	3	34.94 ± 3.32	36.90 ± 1.82	NS
HEC-1B - 36 Hours				
Live Cells	3	74.90 ± 8.21	68.55 ± 6.14	NS
Dead Cells	3	25.11 ± 1.21	31.46 ± 2.12	NS

^aData are expressed as mean live cells or dead cells in normoxia or hypoxia ± SE

^bN represents the number of samples per condition

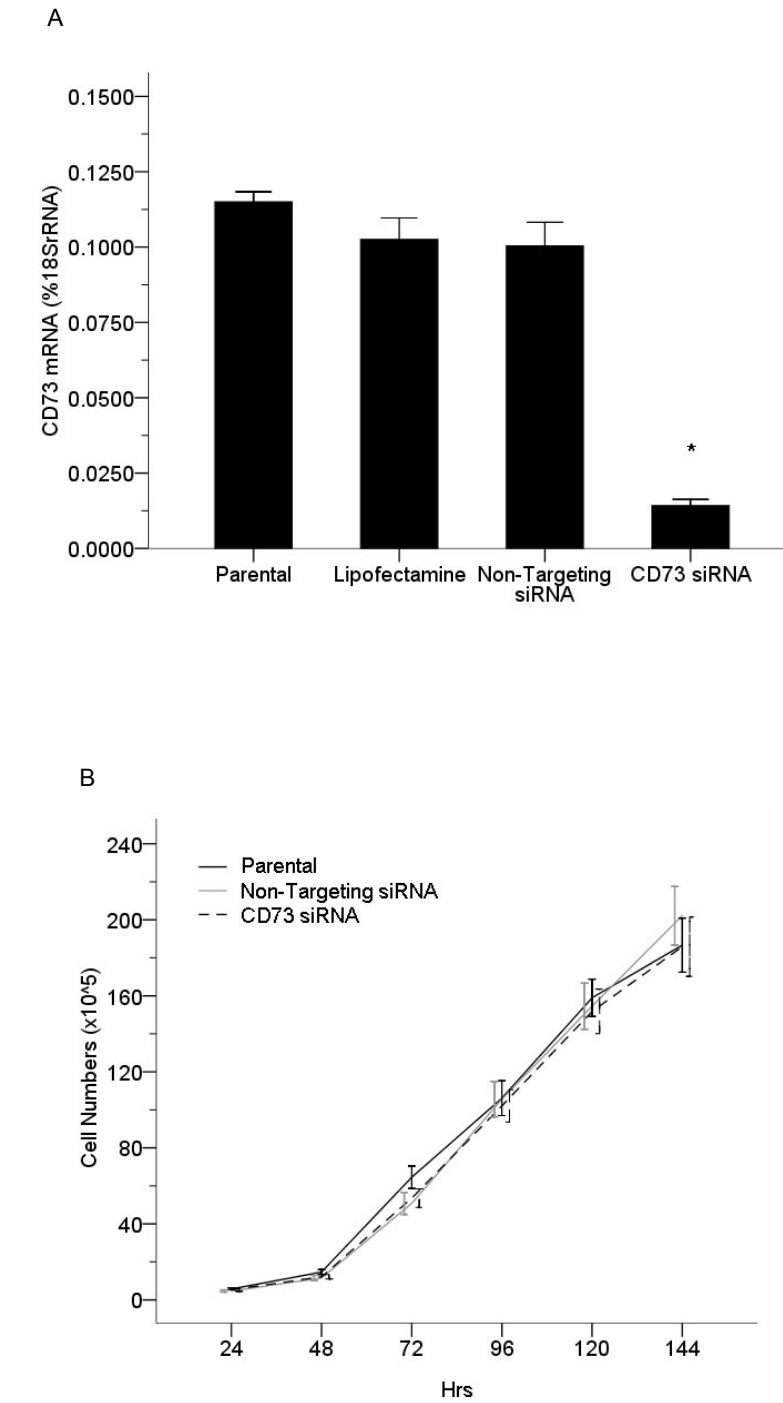
Silencing CD73 by siRNA significantly increases the migration and invasion of hypoxic endometrial carcinoma cells, HEC-1A and HEC-1B

Hypoxia significantly inhibits the migration and invasion of CD73 expressing endometrial carcinoma cells. To determine if CD73 was responsible for the inhibiting effects we observed, CD73 expression was inhibited in the endometrial carcinoma cell lines HEC-1A and HEC-1B by siRNA transfection. siRNA silencing of HEC-1A cells significantly decreased *CD73* transcript levels, at 24 hours post-transcription, to roughly 10% of that of the non-targeting siRNA control (Figure 8.3 A). Proliferation of HEC-1A cells was unaffected by silencing CD73 (Figure 8.4 B). Similar to *CD73* transcript levels, CD73's protein expression was significantly lower, by 72 hours post-transfection, in HEC-1A cells treated with siRNA compared to the non-targeting siRNA control (Figure 8.3 C). In the two-chamber migration and invasion studies, CD73 silencing significantly affected the migration and invasion of the endometrial carcinoma cells. As shown in Figure 8.4 A, HEC-1A cells targeted with CD73 siRNA exhibited increased cell migration as compared to non-targeting siRNA control treated cells. Following CD73 silencing by siRNA, an increase in cell migration (Figure 8.4 B) and invasion (Figure 8.4 C) was observed in HEC-1B cells, as compared to non-targeting siRNA control treatments. Data for Figure 8.4 are summarized in Table 8.3. These data suggest that the cell migration and invasion inhibitory effects initially observed with hypoxia are due, at least in part, to CD73.

Figure 8.3. Effects of CD73 silencing by siRNA in endometrial carcinoma cells, HEC-1A.

(A) *CD73* transcript expression in HEC-1A cells was assessed by qRT-PCR following incubation with non-targeting siRNA or 1247 *CD73* siRNA. *CD73* transcript levels were significantly suppressed by 24 hours compared to the control groups. (B) HEC-1A cell proliferation following incubation with non-targeting siRNA or 1247 *CD73* siRNA. Proliferation remained unchanged with *CD73* silencing. (D) *CD73* immunofluorescence in HEC-1A cells following incubation with non-targeting siRNA or 1247 *CD73* siRNA. *CD73* protein was reduced in the *CD73* siRNA treated cells. Error bars represent ± 1 SE. Image magnification 10X . (* $p < 0.05$)

Figure 8.3



C

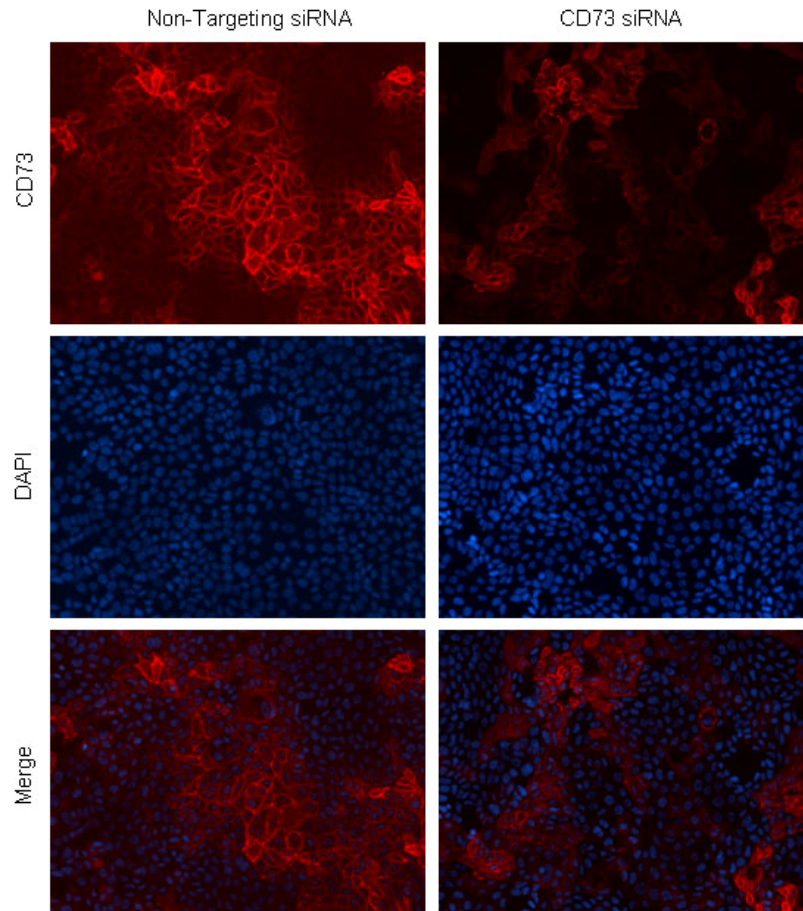
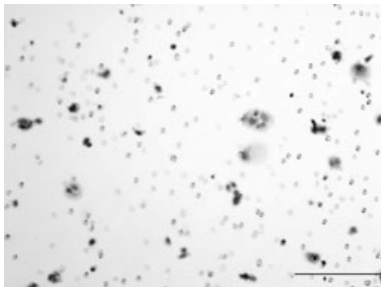
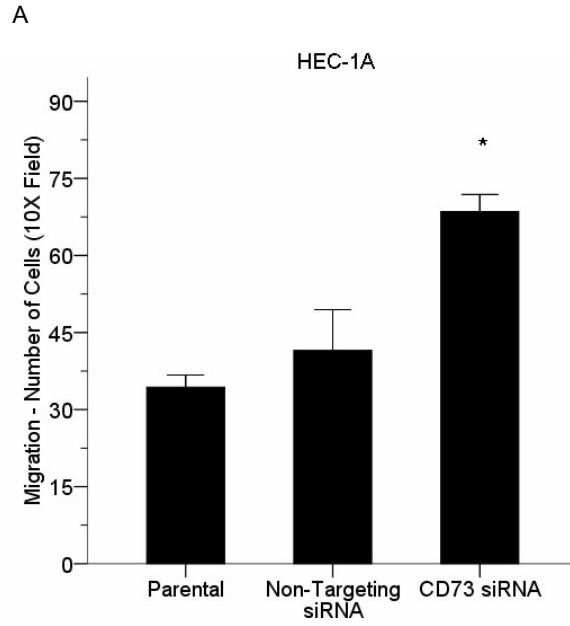
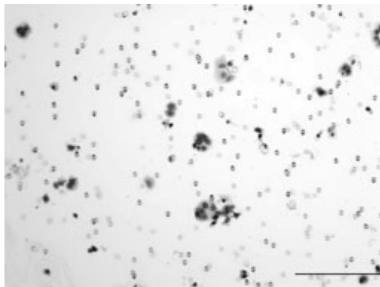


Figure 8.4. HEC-1A migration and HEC-1B migration and invasion in hypoxia following CD73 silencing by siRNA. CD73 was silenced by transfection of CD73 siRNA in endometrial carcinoma cells, HEC-1A and HEC-1B. Following manipulation, cells were placed in hypoxic (1% O₂, 5% CO₂) conditions along with 50μM of 5'AMP. Migration and invasion was then studied *in vitro*. (A) HEC-1A migration. Silencing of CD73 by siRNA significantly increased HEC-1A migration. (B) HEC-1B migration and (C) invasion. Silencing of CD73 by siRNA significantly increased HEC-1B migration and invasion. Representative images of cell migration and invasion are shown alongside graphs. Error bars represent ± 1 SE. Image magnification 10X. (*p<0.05)

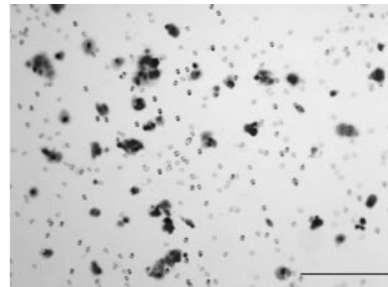
Figure 8.4



Parental

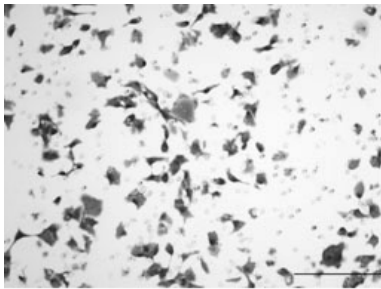
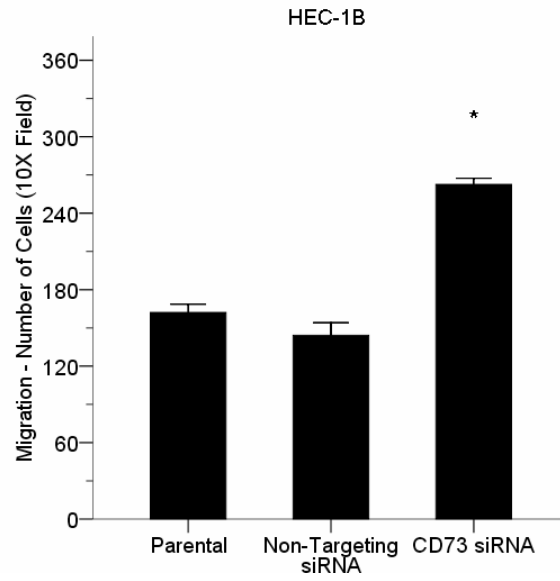


Non-Targeting siRNA

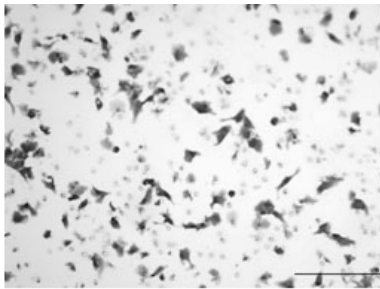


CD73 siRNA

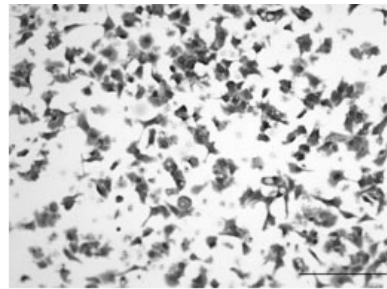
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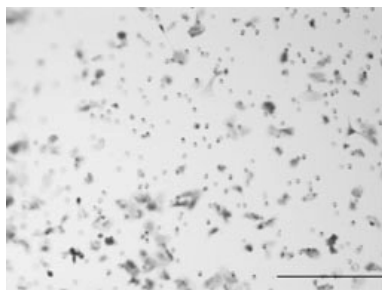
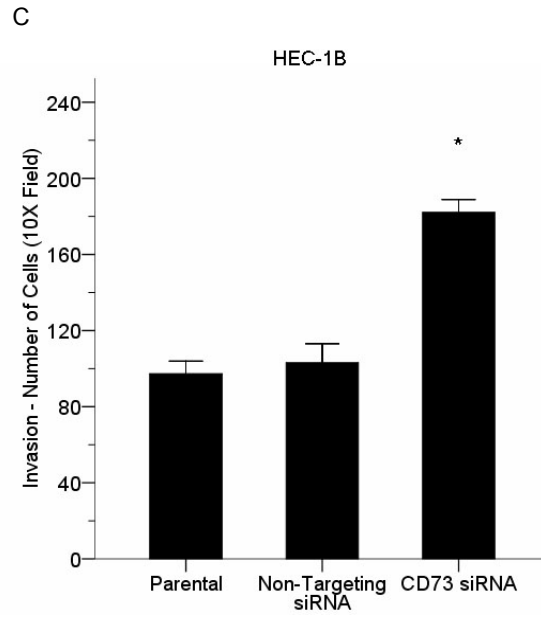
Parental



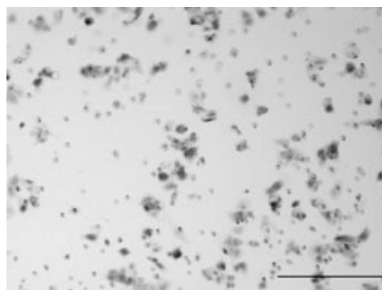
Non-Targeting siRNA



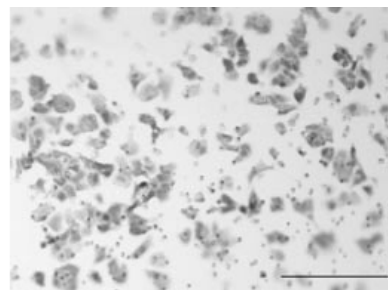
CD73 siRNA



Parental



Non-Targeting siRNA



CD73 siRNA

Table 8.3. HEC-1A migration and HEC-1B migration and invasion in hypoxia following CD73 silencing by siRNA – Data summary from Figure 8.4

	bN	aMean ± SE	p value (vs non-targeting siRNA)
HEC-1A - Migration			
Parental	3	34.37 ± 2.35	NS
Non-Targeting siRNA	3	41.52 ± 7.93	--
CD73 siRNA	3	68.56 ± 3.30	0.001
HEC-1B - Migration			
Parental	3	162.19 ± 6.35	NS
Non-Targeting siRNA	3	144.19 ± 9.89	--
CD73 siRNA	3	262.67 ± 4.77	0.00000005
HEC-1B - Invasion			
Parental	3	97.31 ± 6.64	NS
Non-Targeting siRNA	3	103.13 ± 9.96	--
CD73 siRNA	3	182.15 ± 6.66	0.0000007

^aData are expressed as mean cell number of migrated or invaded cells in a 10X field ± SE

^bN represents the number of migration or invasion inserts per condition

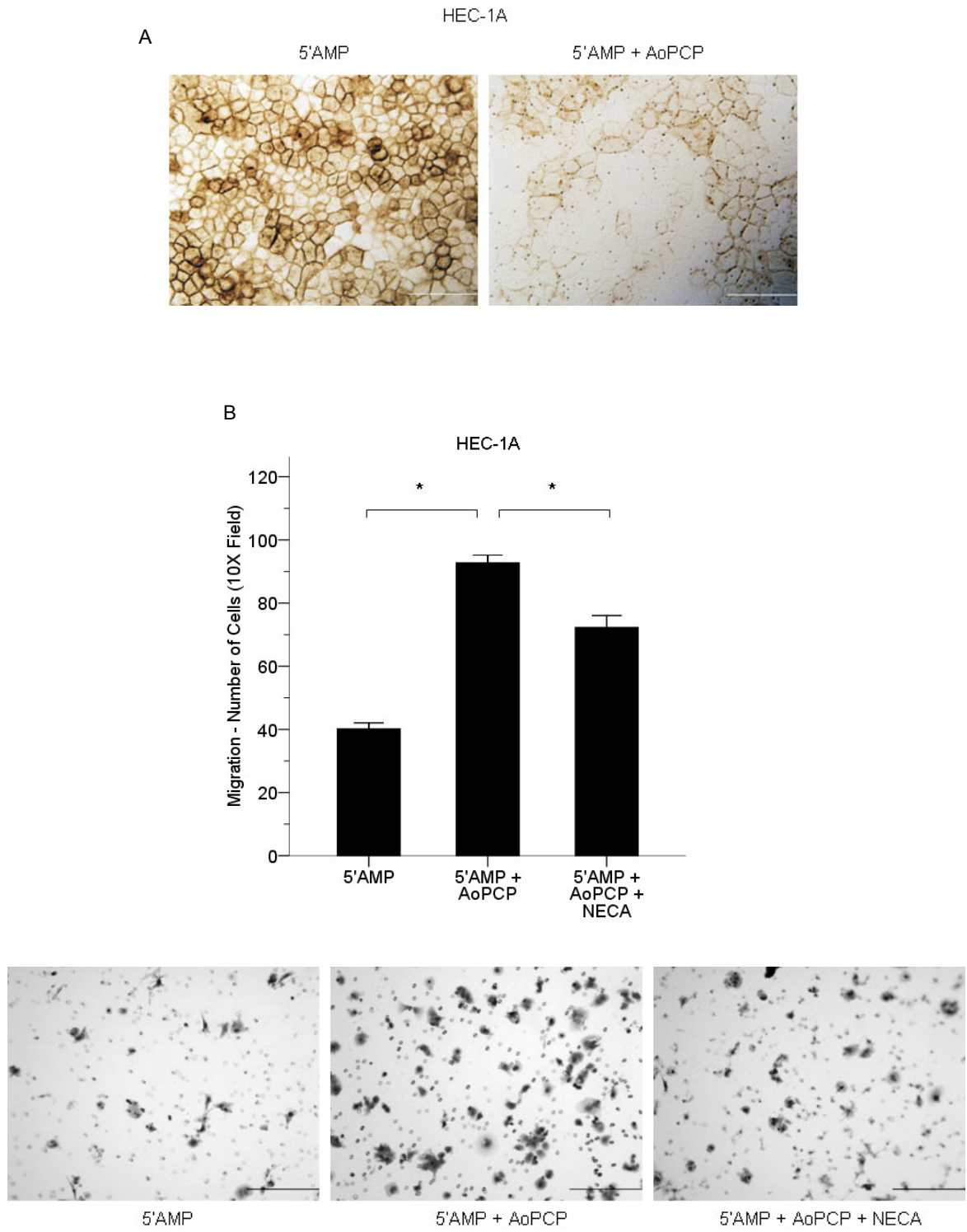
Pharmacological blockade of CD73's catalytic activity increases migration and invasion of endometrial carcinoma cells, HEC-1A and HEC-1B

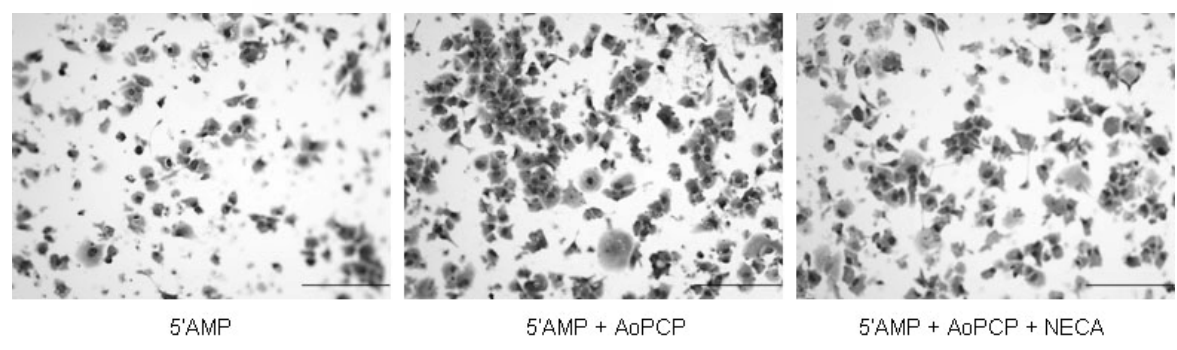
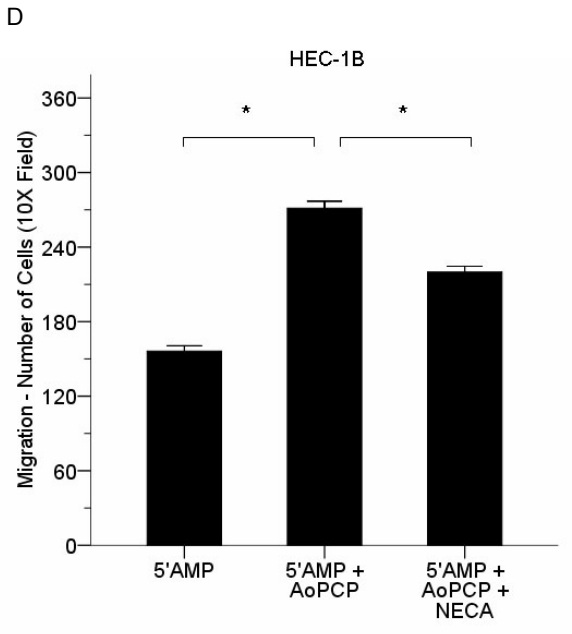
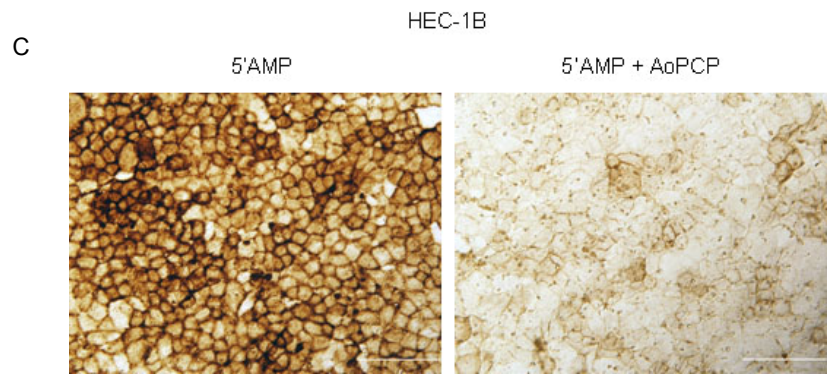
CD73 has been described to have catalytic (reviewed, (157)) and non-catalytic functions. Non-catalytic functions include serving as a co-receptor for T-cell activation (158-162) and a binding partner for ECM proteins (163-165). Targeting CD73 by siRNA (Figure 8.4) does not indicate whether the catalytic function of CD73 is important in inhibiting EC cell migration and invasion or whether it involves a non-catalytic function. Therefore, HEC-1A and HEC-1B migration and invasion were assessed in the presence of a specific catalytic inhibitor for CD73, α,β -methylene diphosphate (AoPCP) (147, 294, 295). AoPCP is a widely used competitive inhibitor for CD73 and has been demonstrated to be very powerful, with a $K_i = 2\text{nM}$ (294); in the present study $100\mu\text{M}$ AoPCP efficiently eliminated CD73's catalytic activity. Inhibition of CD73 by AoPCP can be demonstrated by enzyme histochemistry. Representative images are shown in Figure 8.5 A (HEC-1A) and C (HEC-1B). Inhibiting CD73's catalytic activity significantly increased HEC-1A migration (Figure 8.5 B) and HEC-1B migration (Figure 8.5 D) and invasion (Figure 8.5 E). These important experiments demonstrate that CD73's effects on inhibiting EC cell migration and invasion are due to its catalytic production of adenosine. CD73-generated adenosine has several possible fates. In addition to activating adenosine receptor subtypes, adenosine can be involved in metabolic activities of the cell. To determine if CD73's generation of adenosine is inhibiting EC cell migration and invasion by activation of adenosine receptor subtypes, the stable adenosine analog and adenosine receptor subtype agonist 5'-N-ethylcarboxamidoadenosine (NECA) was employed. The addition of $10\mu\text{M}$ NECA reversed the effect of AoPCP on endometrial carcinoma cells. HEC-1A (Figure 8.5 B) and HEC-1B (Figure 8.5 D) cell migration significantly decreased, approximately by 50%, compared to AoPCP treated cells. HEC-1B cell invasion was completely reduced back to the invasion levels seen in 5'AMP treated cells (Figure 8.5 E). A data summary of HEC-1A and HEC-1B migration and invasion

studies with 5'AMP, 5'AMP and AoPCP, and 5'AMP and AoPCP and NECA is provided in Table 8.4. Importantly, these studies have used physiologically relevant concentrations for 5'AMP (50µM), AoPCP (100µM), and NECA (10µM). Collectively, these data suggest that CD73's inhibitory effect on EC cell migration and invasion occurs via activation of an adenosine receptor subtype.

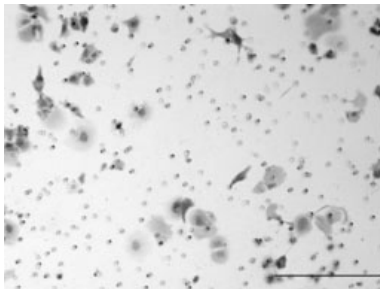
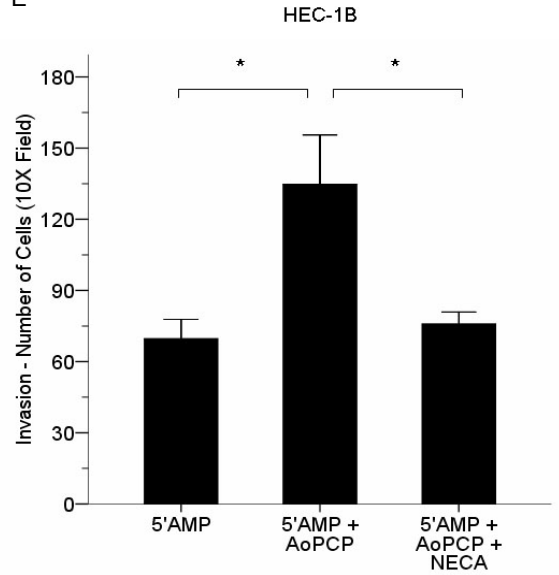
Figure 8.5. HEC-1A migration and HEC-1B migration and invasion in hypoxia following catalytic inhibition of CD73 and application of an adenosine analog, NECA. Endometrial carcinoma cells, HEC-1A and HEC-1B, were treated with 50 μ M 5'AMP or 50 μ M 5'AMP and 100 μ M of the CD73 catalytic inhibitor, α , β -methylene diphosphate (AoPCP). 10 μ M of the stable adenosine analog, 5'-N-ethylcarboxamidoadenosine (NECA), were added to 50 μ M 5'AMP and 100 μ M AoPCP samples to determine if adenosine activation of adenosine receptors inhibited HEC-1A and HEC-1B migration and invasion. Migration and invasion were assessed in hypoxic (1% O₂, 5% CO₂) conditions. (A) Catalytic inhibition of CD73 with AoPCP significantly increased HEC-1A migration. With addition of NECA, the migration of HEC-1A was partially inhibited. AoPCP significantly increased HEC-1B migration (B) and invasion (C). With addition of NECA, the migration and invasion of HEC-1B were partially (migration) or completely (invasion) restored to that seen with 5'AMP. Representative photomicrographs of cell migration and invasion are shown alongside graphs. Error bars represent \pm 1 SE. Image magnification 10X. (*p<0.05)

Figure 8.5

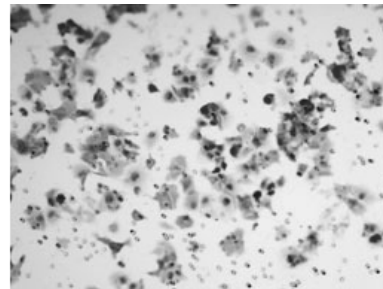




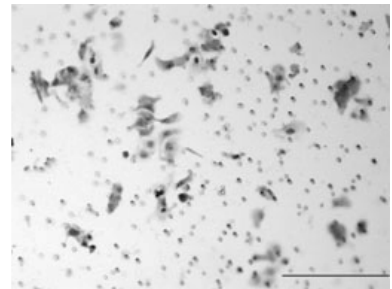
E



5'AMP



5'AMP + AoPCP



5'AMP + AoPCP + NECA

Table 8.4. HEC-1A migration and HEC-1B migration and invasion in hypoxia following catalytic inhibition of CD73 and application of an adenosine analog, NECA – Data summary from Figure 8.5

	^bN	^aMean ± SE	p value (vs 5'AMP)	p value (vs 5'AMP + AoPCP)
HEC-1A - Migration				
5'AMP	3	40.22 ± 1.86	--	0.000000005
5'AMP + AoPCP	3	92.74 ± 2.46	0.000000005	--
5'AMP + AoPCP + NECA	3	72.32 ± 3.71	0.000000005	0.000008
HEC-1B - Migration				
5'AMP	3	156.33 ± 4.25	--	0.000000005
5'AMP + AoPCP	3	271.30 ± 5.61	0.000000005	--
5'AMP + AoPCP + NECA	3	219.96 ± 4.64	0.000000005	0.000000005
HEC-1B - Invasion				
5'AMP	3	69.73 ± 8.07	--	0.002
5'AMP + AoPCP	3	134.80 ± 20.75	0.002	--
5'AMP + AoPCP + NECA	3	75.93 ± 5.00	NS	0.005

^aData are expressed as mean cell number of migrated or invaded cells in a 10X field ± SE

^bN represents the number of migration or invasion inserts per condition

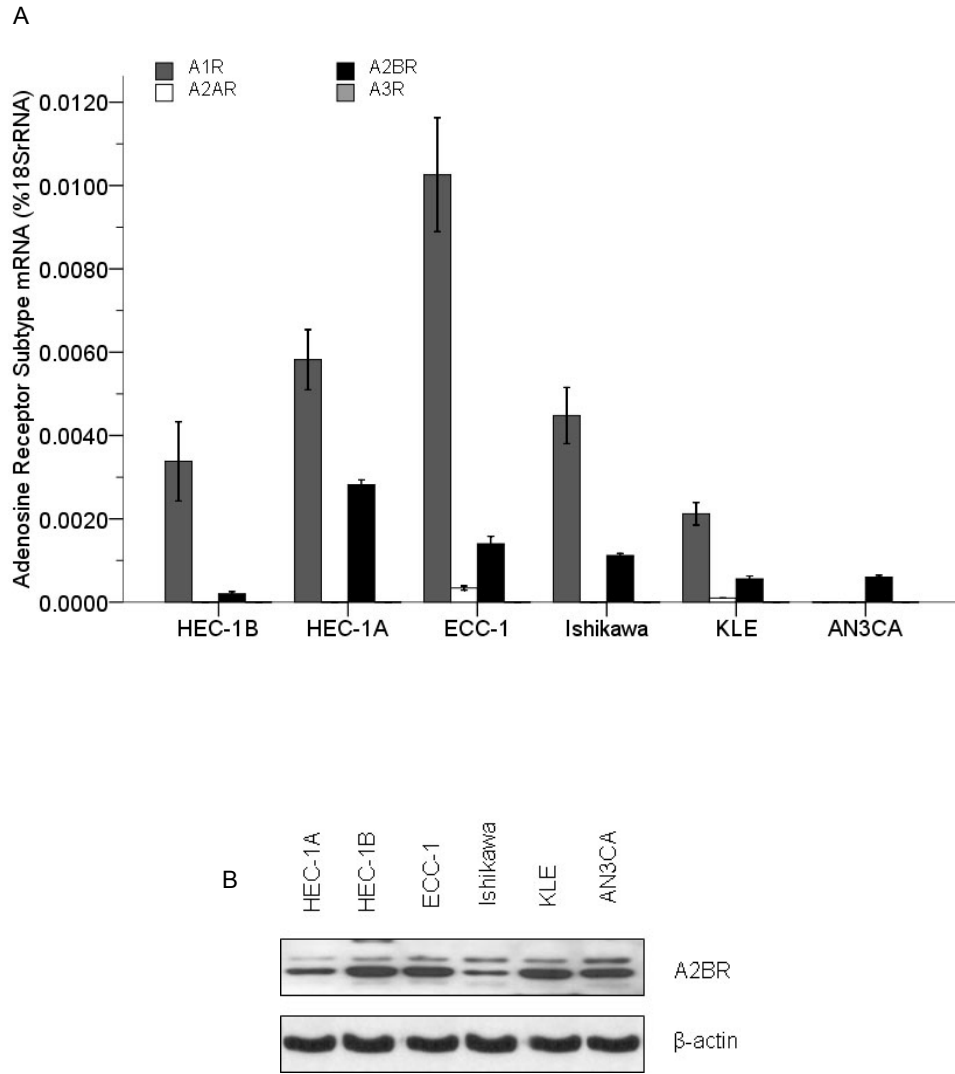
Antagonism of the adenosine receptor subtype, A2BR, increases the migration and invasion of endometrial carcinoma cells, HEC-1A and HEC-1B

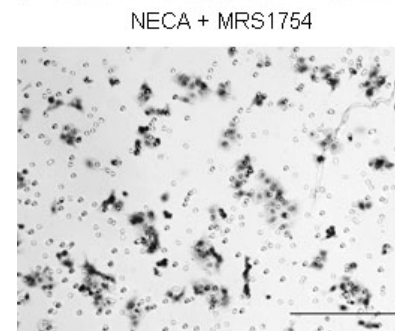
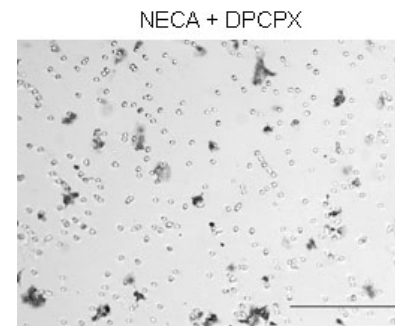
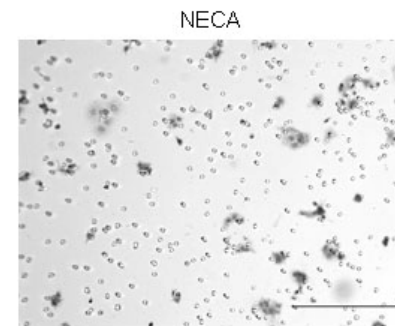
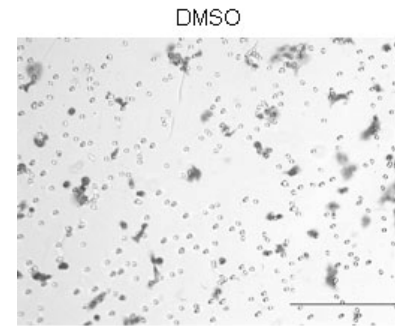
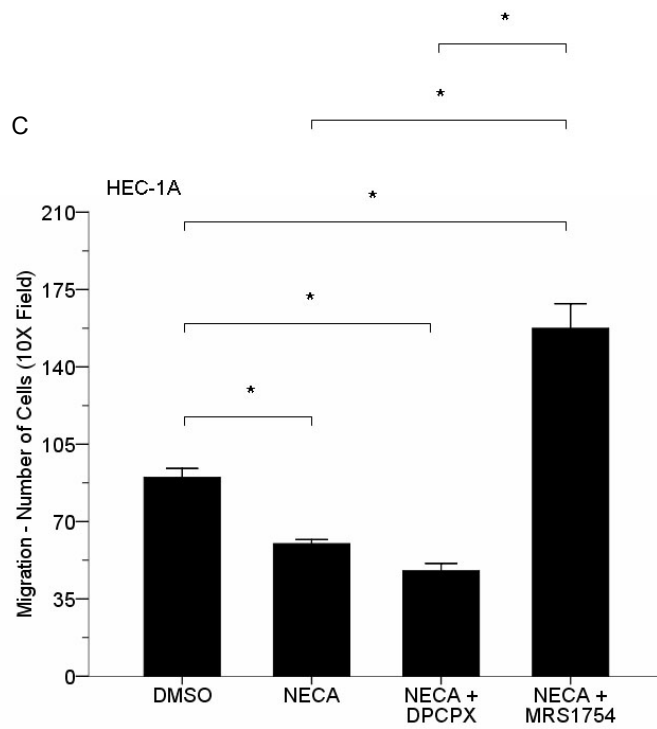
NECA is a non-selective adenosine receptor agonist, so it is unknown which adenosine receptor subtype(s) adenosine is activating in order to inhibit EC cell migration and invasion (Figure 8.5). Fortunately, a variety of selective pharmacological agonists and antagonists to the adenosine receptor subtypes are commercially available. These agents have proven to be important tools in detailing the function of a certain adenosine receptor subtype (179). We first were interested in determining which adenosine receptor subtypes were expressed in our endometrial carcinoma cells. Figure 8.6 (A) shows the entire panel of endometrial carcinoma cells we assessed by qRT-PCR for transcripts levels of the four adenosine receptor subtypes, A1R, A2AR, A2BR, and A3R. A1R and A2BR are the prominent adenosine receptor subtypes expressed in all endometrial carcinoma cells, including HEC-1A and HEC-1B. Detection of the adenosine receptor subtypes by immunoblot has been a challenge due to the lack of antibodies that recognize specific adenosine receptor subtypes. An immunoblot for A2BR is shown in Figure 8.6 B. The dual band at approximately 50kDa is consistent with data reported in the literature (296). We next chose to evaluate the migration and invasion of HEC-1A and HEC-1B in response to treatment with NECA and a selective A1R or A2BR antagonist. The utility of combining the non-selective agonist NECA along with highly selective antagonists has been demonstrated (reviewed, (179)). DPCPX was utilized to antagonize A1R. DPCPX is 20-fold more selective for A1R than A2BR (297). For A2BR antagonism, MRS1754 was employed. MRS1754 is 400-fold more selective for A2BR than A1R (298). As anticipated, for HEC-1A (Figure 8.6 C) and HEC-1B (Figure 8.6 D) migration assays NECA significantly reduced the migration of the cells compared to the vehicle treated control, DMSO. Antagonism of the A1R by DPCPX had no great effect on the migration of HEC-1A or HEC-1B cells compared to NECA. However, A2BR antagonism significantly increased HEC-1A and HEC-1B migration. Similar effects were found for HEC-1B

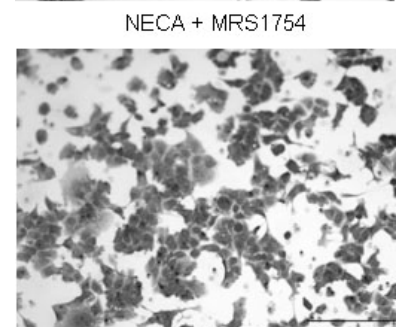
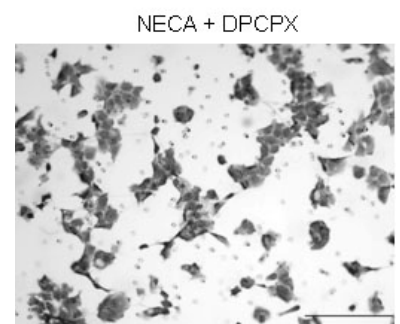
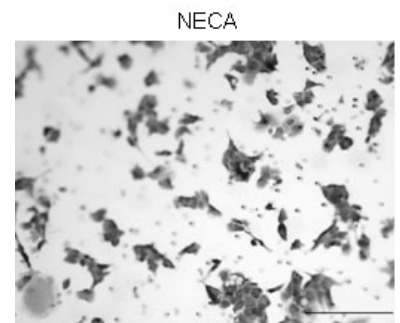
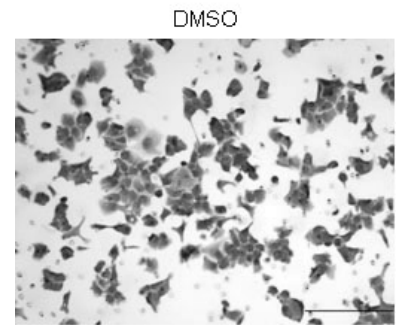
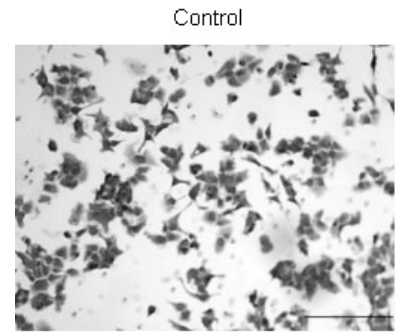
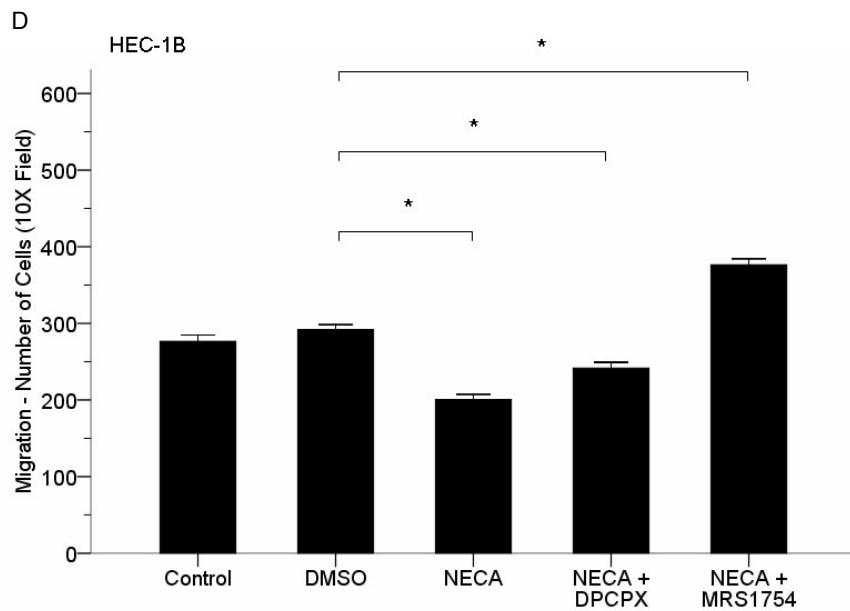
invasion (Figure 8.6 E). These studies indicate that adenosine generation by CD73 and adenosine's activation of A2BR are the mechanisms involved in inhibiting EC cell migration and invasion. It is important to emphasize that this mechanism of Stressor → CD73 → Adenosine → A2BR is the same as that used for adenosine's mediation of barrier function. It is therefore likely that activation of A2BR by CD73-generated adenosine is inhibiting cell migration and invasion by inducing cell-cell adhesion function.

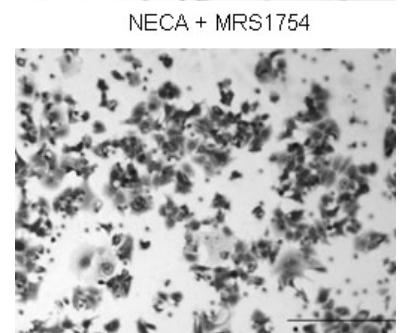
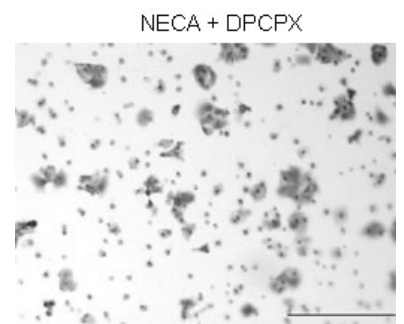
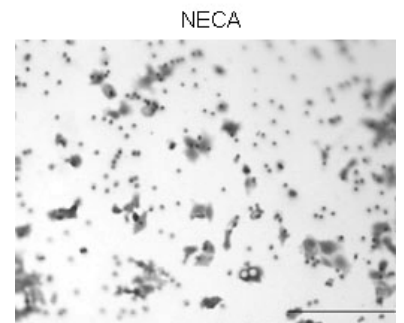
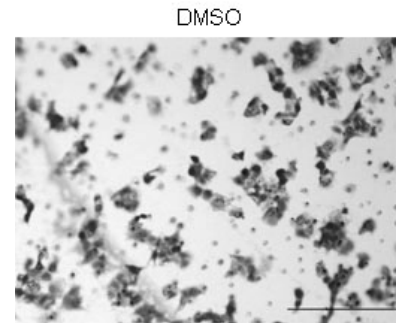
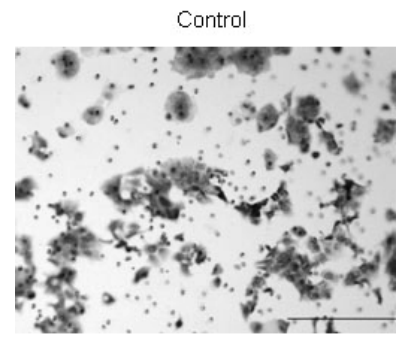
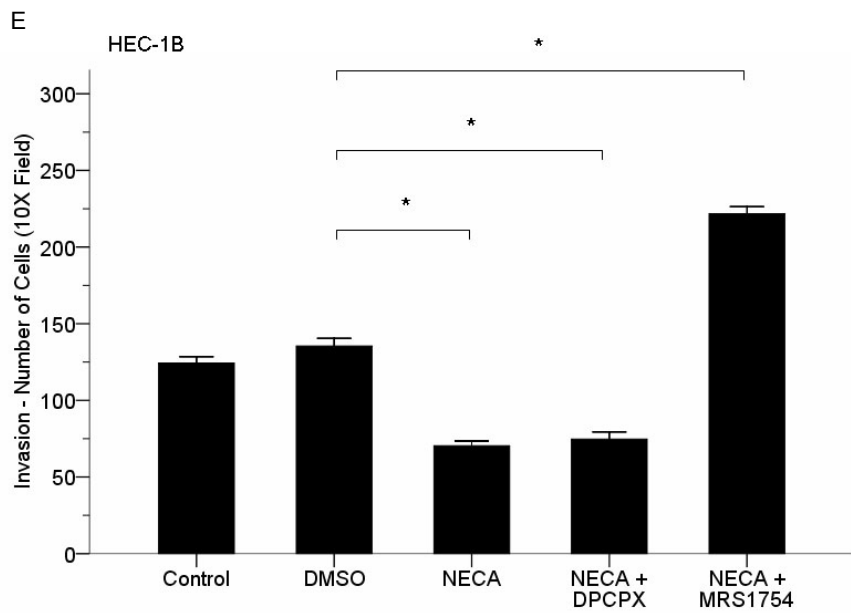
Figure 8.6. HEC-1A migration and HEC-1B migration and invasion in hypoxia following adenosine receptor antagonism. Endometrial carcinoma cells, HEC-1A and HEC-1B, were treated with vehicle control, DMSO, 10 μ M NECA, 10 μ M NECA + 1 μ M adenosine receptor subtype A1R antagonist, DPCPX, or 10 μ M NECA + 1 μ M adenosine receptor subtype A2BR antagonist, MRS1754. Migration and invasion were assessed in hypoxic (1% O₂, 5% CO₂) conditions. (A) qRT-PCR detection of adenosine receptor subtype expression, A1R and A2BR, in HEC-1A and HEC-1B. (B) Immunoblot demonstrating A2BR protein expression. (C) NECA significantly inhibits HEC-1A migration. Antagonism of A1R by DPCPX did not increase HEC-1A migration. A2BR antagonism caused a significant increase in HEC-1A migration. NECA significantly reduced HEC-1B migration (D) and invasion (E). A1R antagonism by DPCPX did not increase HEC-1B migration and invasion. A2BR antagonism caused a significant increase. Representative photomicrographs of cell migration and invasion are shown alongside graphs. Error bars represent \pm 1 SE. Image magnification 10X. (*p<0.05)

Figure 8.6









Summary

The increased ability to migrate and invade are beneficial features to carcinoma cells, especially under the circumstances of unfavorable conditions, such as hypoxia. Generation of adenosine by CD73-expressed carcinoma cells in hypoxic conditions would appear to be a counteractive event in the progression of these EC cells. Such an event would be predicted to result in the induction of barrier function. These data present within this specific aim show CD73's generation of adenosine, and adenosine's activation of A2BR to be functionally responsible for inhibiting the migration and invasion of well-differentiated EC cells.

CHAPTER NINE

Results

Specific Aim 5. Identify the events or proteins of cell-cell adhesion that are regulated by adenosine's activation of A2BR.

Introduction

The gap in knowledge in the adenosine field concerning adenosine's barrier function activity has been the molecular mechanisms by which it regulates cell-cell adhesions. Few studies have examined this topic (220, 221). Maintaining or regaining barrier function is a dynamic event that involves not only the stabilization or membrane-trafficking of integral membrane and peripheral plaque proteins to adhesion structures, but includes actin cytoskeleton remodeling, relaxation of the contracting perijunctional actin-myosin II ring, activation of GTPases, and phosphorylation of tight junction proteins. cAMP, the primary secondary messenger of many G protein-coupled receptors, including A2BR, is central to the activation of many of these intracellular mediators and barrier function events. Previous studies that have provided the most insight into adenosine's activation of A2BR have described a cAMP driven, PKA-dependent phosphorylation of VASP (220, 221). VASP is a member of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) protein family that is involved in regulating actin dynamics (299, 300). VASP is a dual resident of both focal adhesions and cell-cell adhesions. Importantly, as will be introduced in this specific aim, VASP is a key resident of filopodia that drive the development of focal adhesions and cell-cell adhesions.

In previous specific aims, CD73 generated adenosine and A2BR increased endometrial epithelial barrier function, and CD73 was significantly down-regulated in ECs that are moderately- or poorly-differentiated, invasive, and metastatic. Loss of CD73 expression was associated with the loss of the epithelial phenotype of carcinoma cells and gain of cell motility and

invasiveness. Importantly, the mechanism by which adenosine induces barrier function (Stressor → CD73 → Adenosine → A2BR → Barrier Function) is identical to the mechanism that inhibits the migration and invasion of EC cells. Collectively, these results suggest a connection between CD73, adenosine, A2BR, and cell-cell adhesions in endometrial carcinoma cells. This specific aim is particularly important, as we are ultimately interested in the future therapeutic use of targeting the purinergic pathway as a way of restoring cell-cell adhesions in carcinoma cells. To move towards accomplishing this goal, it is important that we first define what cell-cell adhesion events or specific individual proteins comprising cell-cell adhesions are regulated by adenosine's activation of A2BR.

This specific aim introduces the involvement of filopodia in the formation of cell-cell adhesions and identifies a mechanism by which cell-cell adhesive filopodia are regulated by adenosine. Currently, adenosine's induction of filopodia has not been described in any tissue or disease type. Therefore, these experiments elucidate a novel mechanism associating cell-cell adhesions and adenosine's barrier function. The findings described within this specific aim have potential relevance for understanding adenosine's barrier function role in tissues other than the endometrium and diseases other than EC.

CD73 expression in HEC-1A and HEC-1B endometrial carcinoma cells is predominantly confined to membranes that are in direct contact with a neighboring cell

CD73 resides in glycolipid-rich membrane subdomains known as lipid rafts and is anchored by a C-terminal glycosyl phosphatidylinositol (GPI) linkage (154). CD73 has been determined to be localized to both the apical and basolateral membranes of cells with its intensity of expression being higher on the apical membrane (275). In the well-differentiated, epithelial-like endometrial carcinoma cells HEC-1A and HEC-1B, membranous CD73 expression was confined to membranes that were in direct contact with a neighboring cell. A trivial explanation for this is that this is an artifact of 2-D culture techniques. However, when HEC-1A cells were grown on a collagen matrix, this same expression pattern for CD73 was maintained (Figure 9.1 A). CD73's catalytic activity followed this same membrane expression pattern, as catalytic activity was found only on the membranes that were in direct contact with a neighboring cell (Figure 9.2 A); this was not affected by cell confluency (Figure 9.2 A-C). This CD73 expression pattern and catalytic activity localization is interesting because though HEC-1A and HEC-1B cells do not have well-defined apical or basolateral membrane distinctions in standard culture conditions, studies of protein trafficking in non-polarized cells indicate that apical proteins are distributed uniformly on the membrane (301).

To more precisely define the location of CD73's catalytic activity, we took advantage of the electron density nature of the lead precipitate product of the CD73 enzyme histochemistry assays (Figure 6.12, Specific Aim 3). The enzyme histochemistry assay was modified to accommodate examination by transmission electron microscopy. Inspection of images obtained by transmission electron microscopy revealed that the lead precipitate was clearly visible and restricted to membranes that were in direct contact with a neighboring cell (Figure 9.2 D). Importantly, CD73's catalytic activity was found to be on the extracellular surface of the membrane, which is typical of this ecto-enzyme. At lower magnifications (5,000X), it appeared

that the paracellular space had a non-specific accumulation of lead precipitate. However, after inspecting higher magnification images (25,000X and 50,000X) (Figure 9.2 E-F), CD73 catalytic activity was pronounced on finger-like extensions that extended from the cell membrane (Figure 9.2 E). These finger-like extensions appeared to be imbedding themselves into the neighboring cell (Figure 9.2 F). In HEC-1B cells these finger-like extensions, which have characteristics of filopodia, extended between membranes of neighboring cells (Figure 9.3). Filopodia were numerous between cells, and all were found only protruding from cells that had a neighboring cell. These characteristics suggest a “zippering” cell-cell adhesion function for these filopodia. The “zippering” function is important, as it leads to the formation of mature adhesion junctions.

Figure 9.1. CD73 membrane expression in endometrial carcinoma cells, HEC-1A, grown on a collagen matrix. CD73 immunofluorescence in endometrial carcinoma cells, HEC-1A, grown on a collagen matrix. CD73's expression is absent from cell membranes that are not in contact with a neighboring cell (arrow). Na⁺K⁺ ATPase is used as a marker of basolateral membranes. Image magnification 20X.

Figure 9.1

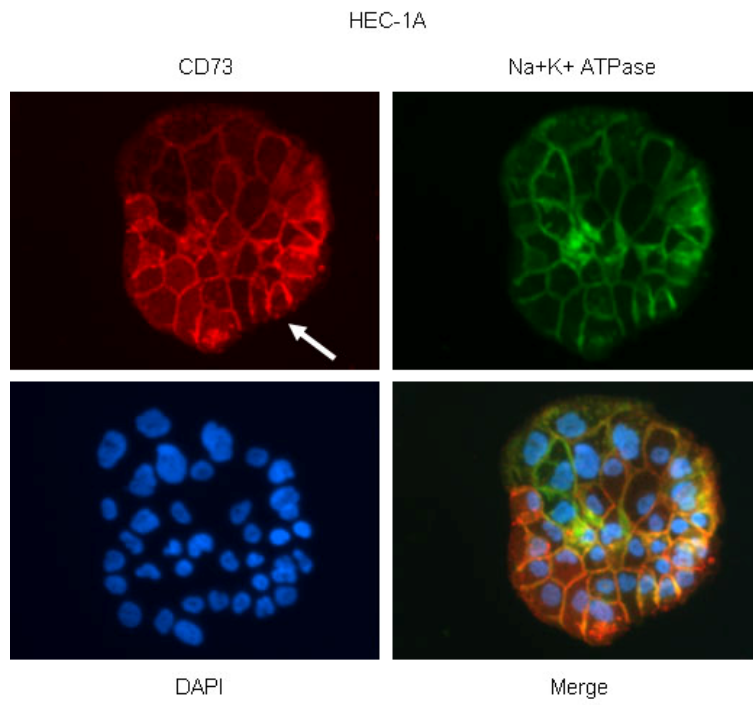
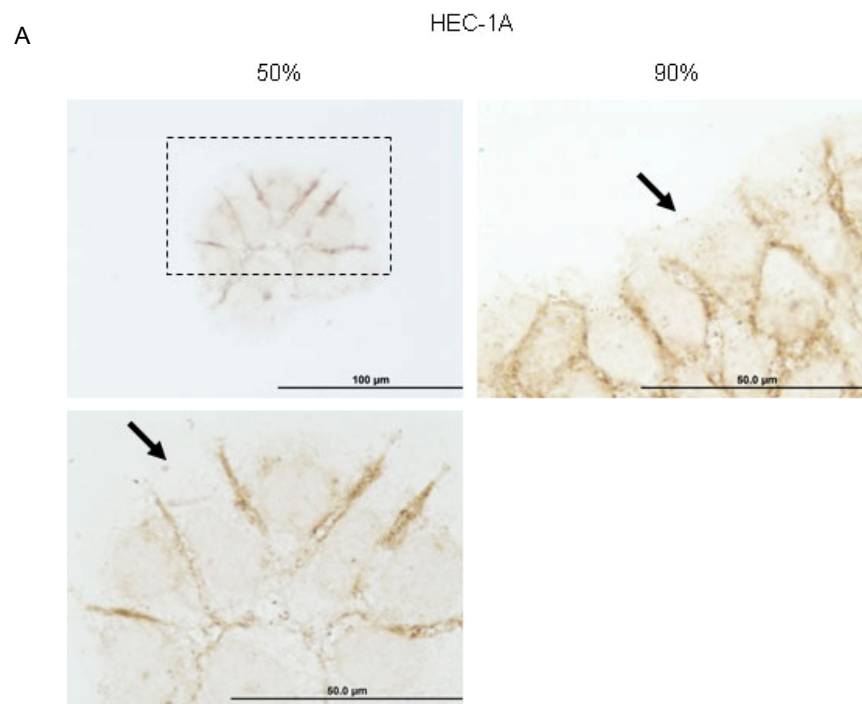


Figure 9.2. Membrane location of CD73's catalytic activity in endometrial carcinoma cells, HEC-1A. (A) CD73 enzyme histochemistry in endometrial carcinoma cells, HEC-1A. (B) Electron photomicrographs of CD73 enzyme histochemistry in HEC-1A. At 50% and 90% confluency, CD73's catalytic activity (brown precipitate) is absent from cell membranes that are not in contact with a neighboring cell (arrow). A higher magnification (40X) of the 50% image is shown. Relative location of the higher magnification is indicated by the inset. (B) Lead precipitate (black) is seen by transmission electron microscopy and is present in the paracellular space of cells. CD73 catalytic activity is low-absent from membranes that are not immediately adjacent to a neighboring cell (arrow). Higher magnification images (25,000X and 50,000X) are shown of the paracellular space. Relative location of the higher magnification is indicated by the inset. Finger-like extensions are found within the paracellular space of HEC-1A cells and show the presence of lead precipitate (black). In the 50,000X magnification, finger-like extensions appear to be imbedding themselves into the neighboring cell (arrow). Image magnification, enzyme histochemistry 20X; enzyme histochemistry magnified image, 40X; electron photomicrographs of enzyme histochemistry, 5,000X, 25,000X, and 50,000X, respectively.

Figure 9.2



B

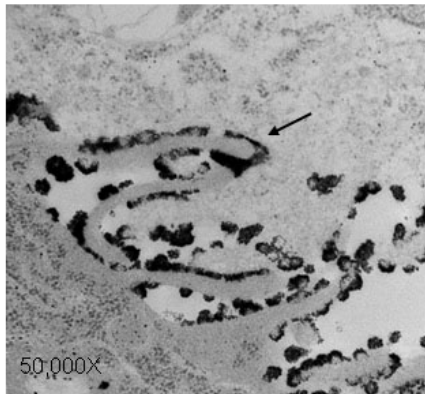
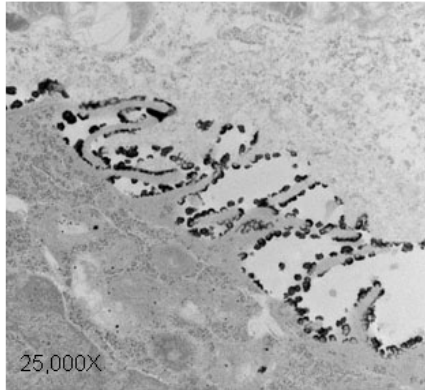
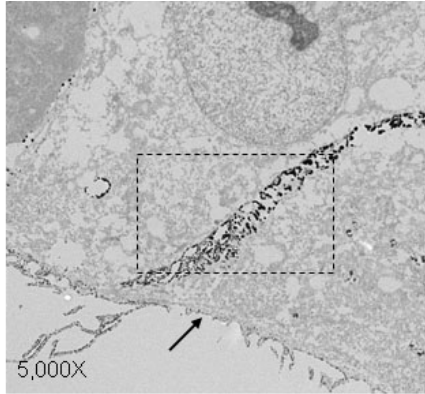
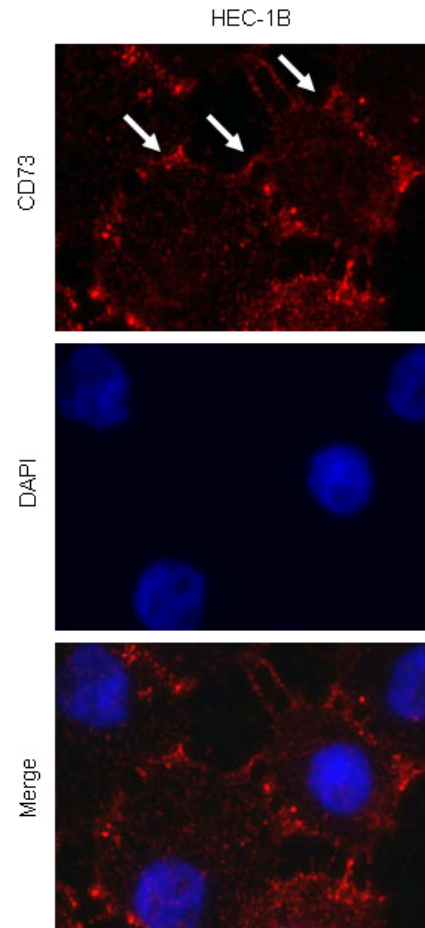


Figure 9.3. CD73 expressing filopodia in endometrial carcinoma cells, HEC-1B. CD73 immunofluorescence in endometrial carcinoma cells, HEC-1B. CD73's intensity is highest in the filopodia extensions between cells (arrows). Filopodia are most numerous between immediately adjacent cells, suggesting a “zippering” cell-cell adhesion function. Filopodia were not observed from membranes not in contact with a neighboring cell. Image magnification 40X.

Figure 9.3

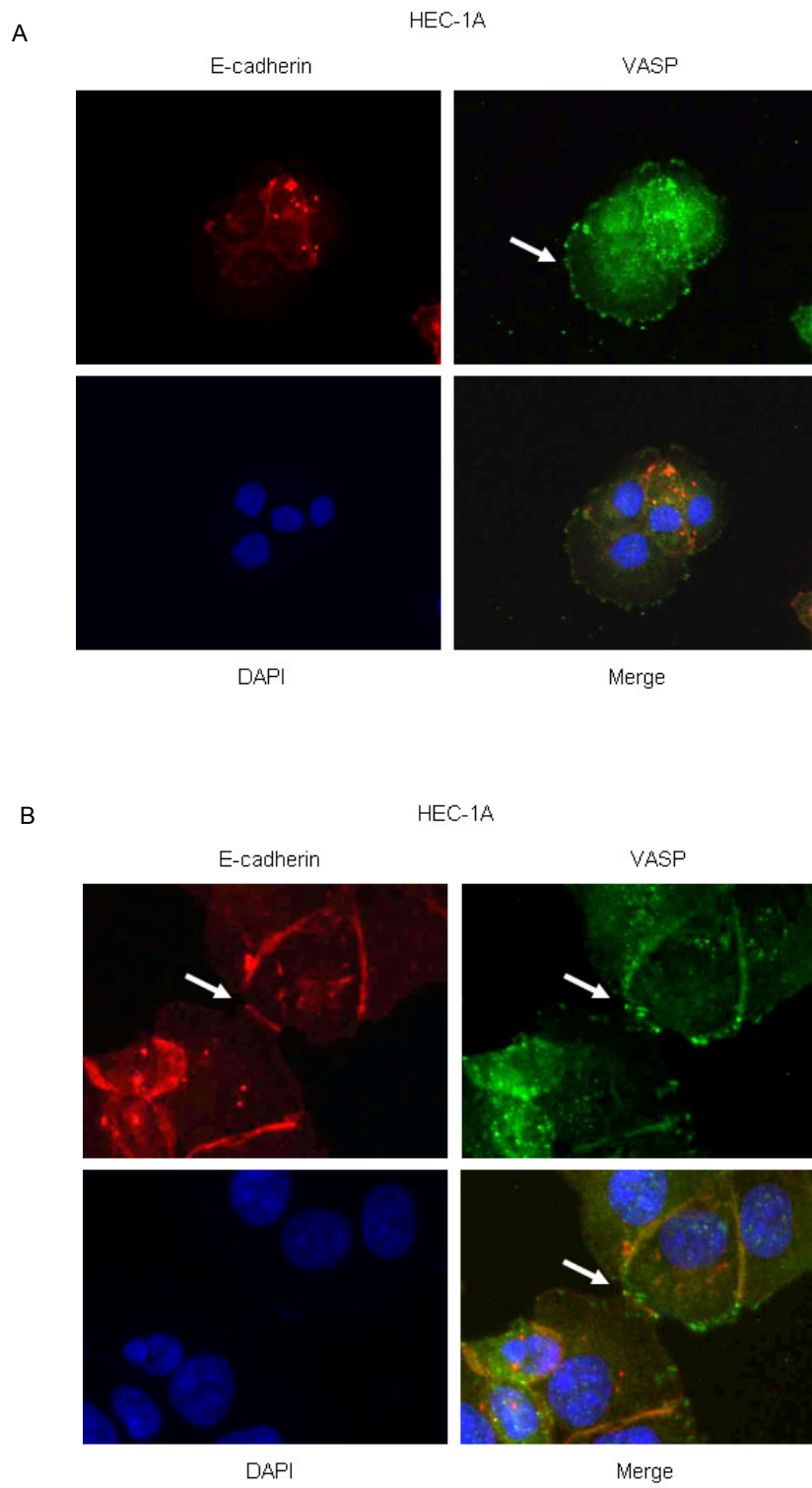


Filopodia that function in cell-cell adhesions are distinct from those that function as focal adhesions.

Filopodia are thin, actin-rich membrane protrusions that have a diverse array of functions, including cell migration (focal adhesions), neurite outgrowth, wound healing, guidance towards chemoattractants, embryonic development, and formation of cell-cell adhesions (reviewed, (302)). The establishment of filopodia in the formation of cell-cell adhesions is well documented in studies of epithelial sheet fusion during embryonic development and wound healing (223-225). Individual cell-cell adhesion formation has been established in cultured keratinocytes (299). The function of filopodia is dependent on one or a combination of events that include the presence of specific proteins in the shafts or tips of filopodia, including E-cadherin (cell-cell adhesions) or integrins (focal adhesions); the presence of structural and signaling proteins in filopodia which respond to external signals (chemoattractant); and the presence of proteins that alter the biochemical and biophysical properties of filopodia (reviewed, (302)). Our observation that CD73's expression and catalytic activity were specific for membranes associated with cell-cell adhesions and that CD73 expressing finger-like extensions (filopodia) were protruding from these cells led us to hypothesize that adenosine and adenosine's activation of A2BR might be involved in filopodia-directed cell-cell adhesion. To first establish that CD73 expressing filopodia were indeed cell-cell adhesive as opposed to those involved in focal adhesions, HEC-1A cells were fluorescently immunostained with the non-specific filopodia marker, VASP, and E-cadherin. VASP is known to localize to regions of dynamic actin reorganization, including the tips of filopodia. VASP promotes actin polymerization by an anti-capping mechanism. As demonstrated in cultured keratinocytes, filopodia with cell-cell adhesion function can be distinguished by their expression of E-cadherin. HEC-1A cells with focal adhesions (Figure 9.4 A) lacked E-cadherin expression. However, newly forming cell-cell adhesions in HEC-1A cells were found to express both VASP and E-cadherin (Figure 9.4 B).

Figure 9.4. Filopodia of focal adhesions and newly forming cell-cell adhesions in endometrial carcinoma cells, HEC-1A. Vascular-stimulated phosphoprotein (VASP) is a marker for filopodia of focal adhesions and newly forming cell-cell adhesions. (A) E-cadherin is absent from VASP positive (arrow) focal adhesion filopodia. (B) E-cadherin and VASP are both localized in the filopodia associated with early cell-cell adhesion formation (arrow). Image magnification (A) 20X and (B) 40X.

Figure 9.4

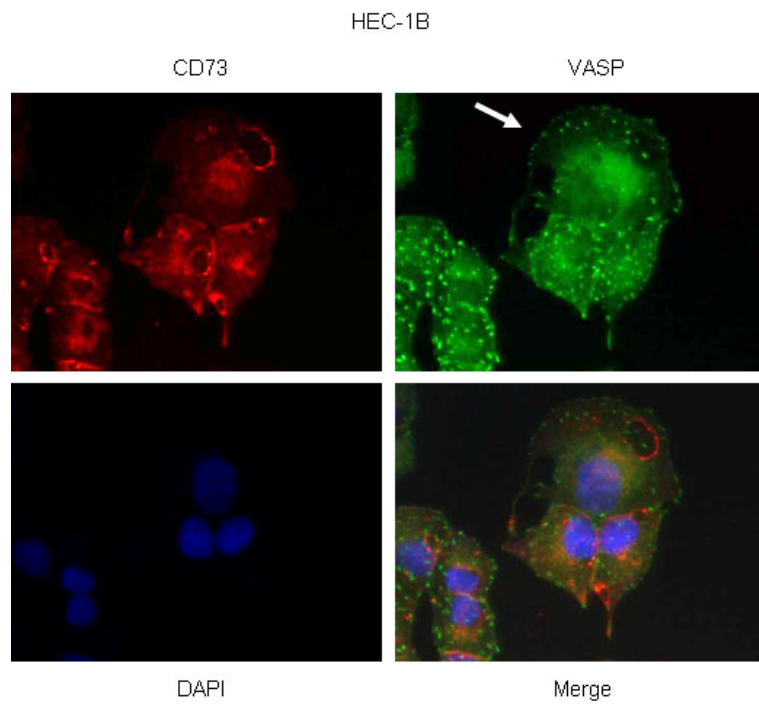
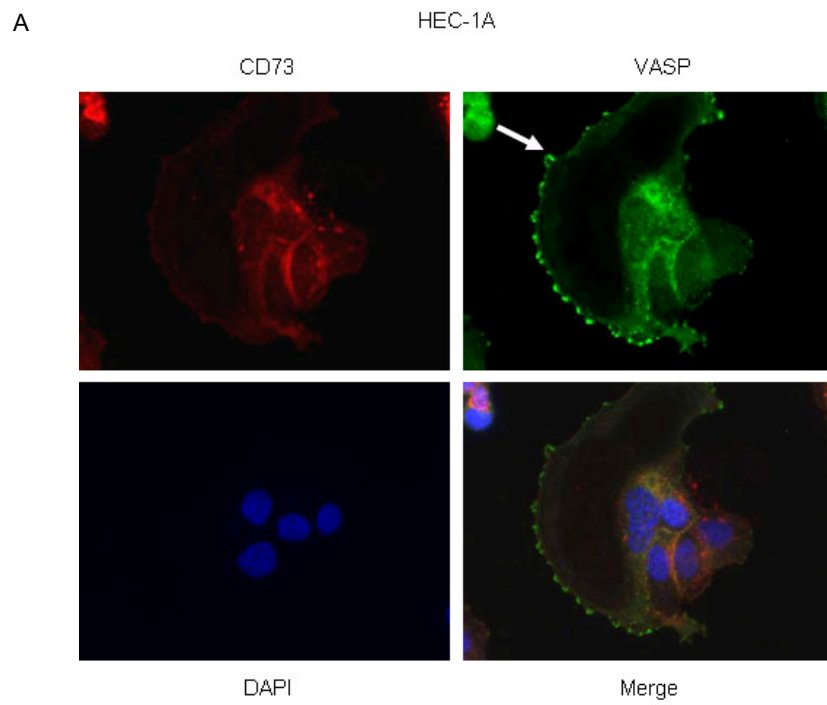


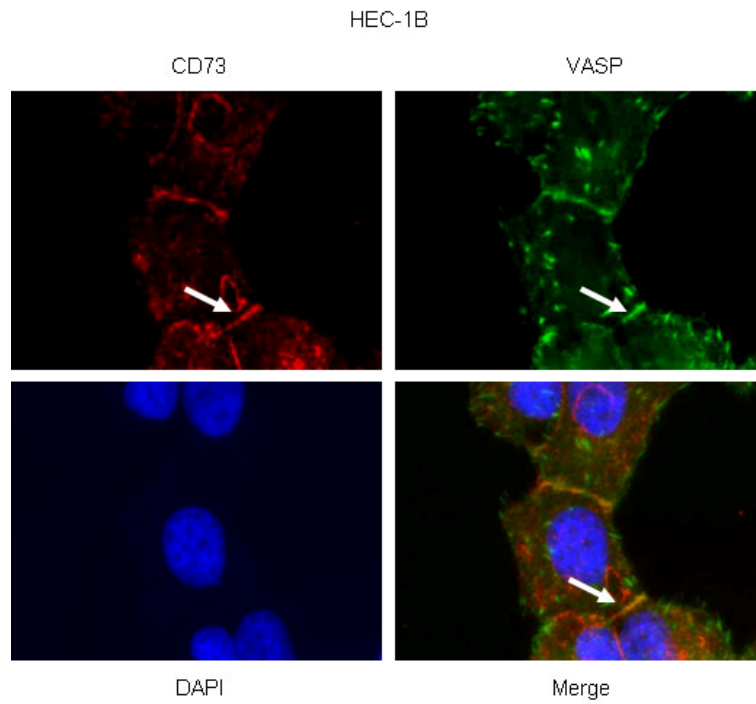
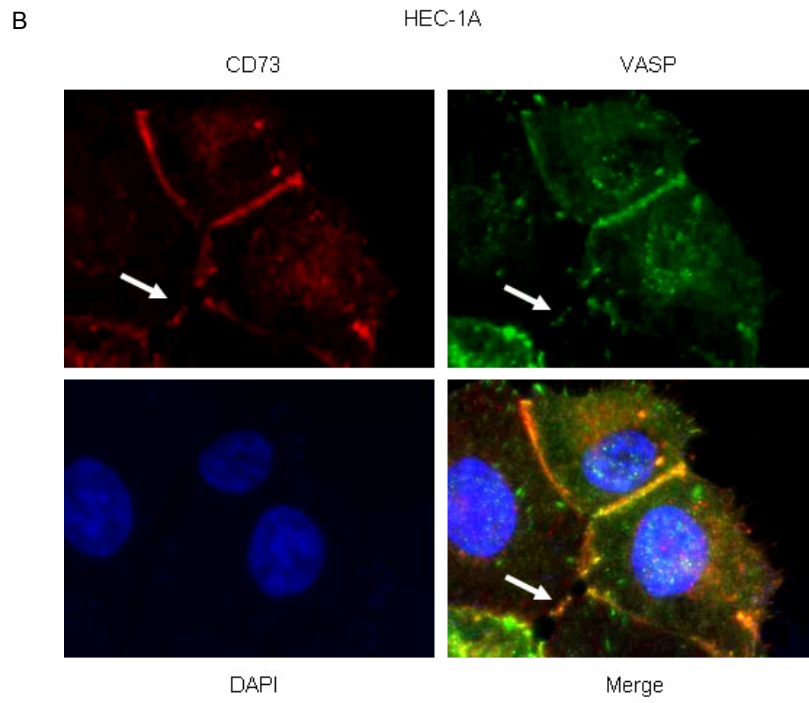
CD73 expression is present in filopodia of newly forming cell-cell adhesions in endometrial carcinoma cells, HEC-1A and HEC-1B

Extensive finger-like extensions extending from membranes of neighboring cells in low to subconfluent conditions were observed. Interestingly, filopodia were not identified in cells lacking direct contact with a neighboring cell. To determine if CD73 was expressed exclusively in filopodia of newly forming cell-cell adhesions (similar to the expression pattern of E-cadherin) HEC-1A and HEC-1B were dual labeled with VASP and CD73. CD73 was absent in VASP positive focal adhesions (Figure 9.5 A). Similar to E-cadherin expression (Figure 9.4 B), CD73 and VASP were localized to the filopodia of newly forming cell-cell adhesions (Figure 9.5 B).

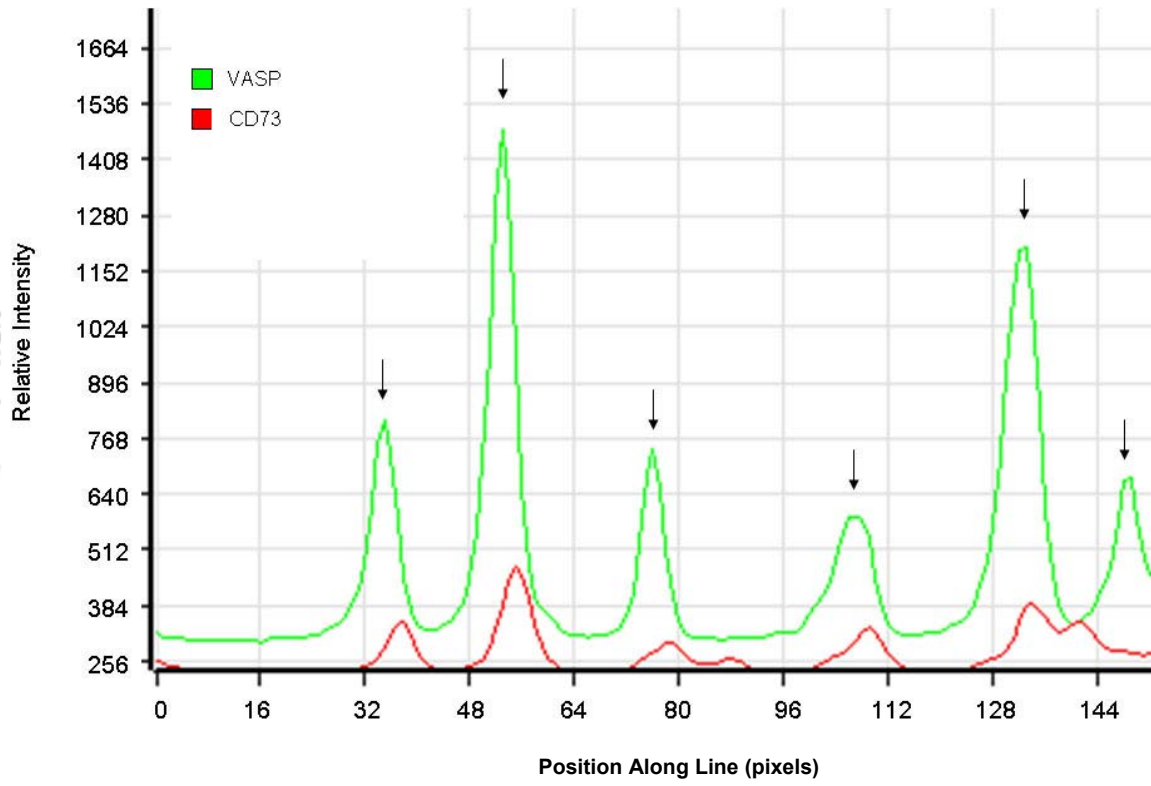
Figure 9.5. CD73's expression in filopodia of newly forming cell-cell adhesions in endometrial carcinoma cells, HEC-1A and HEC-1B. (A) E-cadherin and vascular-stimulated phosphoprotein (VASP) immunofluorescence in endometrial carcinoma cells, HEC-1A and HEC-1B. VASP is a marker for filopodia of focal adhesions and newly forming cell-cell adhesions. (A) E-cadherin is absent from VASP positive (arrow) focal adhesion filopodia. (B) CD73 and vascular-stimulated phosphoprotein (VASP) immunofluorescence in endometrial carcinoma cells, HEC-1A and HEC-1B. CD73 and VASP are detected in the filopodia associated with early cell-cell adhesion formation (arrow). (C) Representative histogram of relative fluorescence intensity of VASP (green) and CD73 (red) in filopodia at cell-cell adhesions. Individual peaks indicate a single filopodium. With each VASP peak a corresponding CD73 peaks is seen. Image magnification (A) 20X; (B) 40X; (C) 60X

Figure 9.5





C



Filopodia and barrier function are increased in HEC-1A endometrial carcinoma cells by adenosine's activation of A2BR

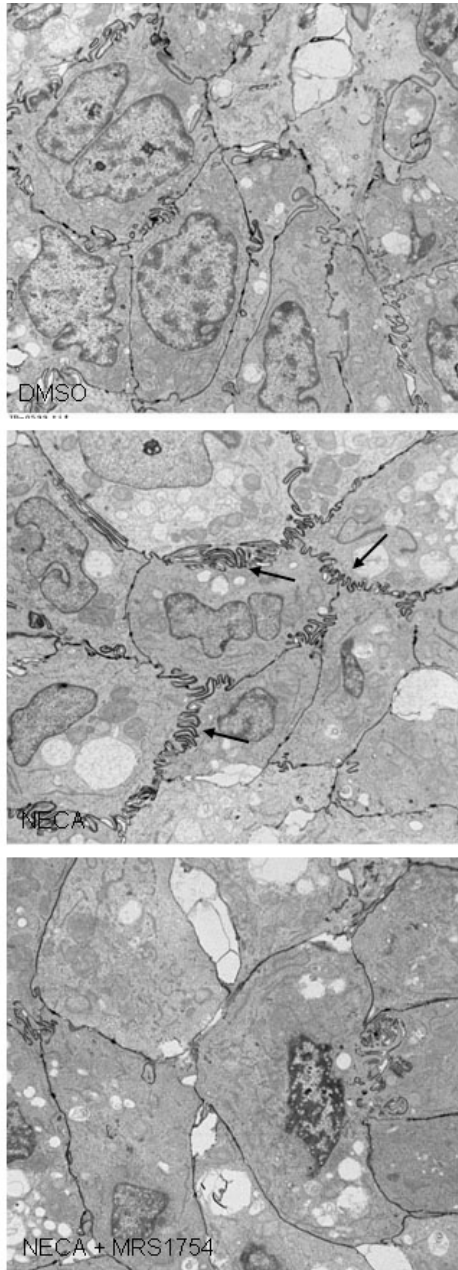
The establishment of cell-cell adhesions between epithelial cells involves the process of actin polymerization and cytoskeleton remodeling. This event is common in many processes requiring cell-cell adhesion, such as embryonic development, wound healing, and steady-state dynamics of cell-cell adhesion remodeling. In embryonic development and wound healing direct actin polymerization and its process of forming cell-to-cell extending filopodia is the driving force for the formation of cell-cell adhesions. Interdigitating filopodia are necessary to properly align and establish initial cell-cell adhesions (223-225). This event, known as adhesion “zippering”, also occurs in cultured keratinocytes (299). Previous observations of CD73's high membrane expression in well-differentiated endometrial carcinoma cells, HEC-1A, however absent in poorly-differentiated (AN3CA) endometrial cancer cell lines (Figure 7.4, Specific Aim 3) suggested the possibility that adenosine's activity is an important regulator of cell-cell adhesions. Studies in this specific aim provide further evidence to support this assumption and include the following: 1.) CD73 is expressed only on cell membranes involved in cell-to-cell contact; 2.) CD73's expression and catalytic activity localized to filopodia that extend from cell-to-cell; 3.) in subconfluent cultures distinct CD73 expressing filopodia can be seen extending from cell-to-cell; and 4.) CD73 expression is limited to filopodia involved in cell-cell adhesion function. Barrier disruption is associated with the loss of cell-cell adhesions and the necessity of having to re-establish these adhesions to maintain homeostasis. We therefore hypothesized that adenosine directs a filopodia-driven cell-cell adhesion event that helps to re-establish cell-cell adhesions in barrier disrupted epithelial cells. To test this hypothesis, HEC-1A monolayers were treated with the adenosine analog, NECA, NECA and the A2BR antagonist, MRS1754, or vehicle control, DMSO. Cell-cell adhesion filopodia were examined using images obtained by transmission electron microscopy. To induce the disruption of cell-cell adhesions, the HEC-1A monolayers were placed in hypoxia

(1% O₂, 5% CO₂) for 36 hours. In vehicle control treated cells (DMSO), a limited number of filopodia were seen among cells (Figure 9.6 A). However, with NECA treatment we found the presence of filopodia to be noticeably higher among cells (Figure 9.6 A). Antagonism of the A2BR blocked the NECA-induced increase in filopodia (Figure 9.6 A). These results suggest that adenosine activation of A2BR is important for the increased formation of filopodia between cells following a hypoxic insult. Interestingly, filopodia length increased when the neighboring cell was farther away (Figure 9.6 B). Also, from these images, filopodia were observed to interdigitate among each other, suggesting an adhesion “zippering” action (Figure 9.6 B). Moreover, filopodia extending from cell-to-cell also appeared to be imbedding themselves into the cytoplasm of the neighboring cell (Figure 9.6 B). To demonstrate that functionally these HEC-1A monolayers are indeed more adhesive and are attempting to preserve barrier function via adenosine signaling through A2BR, paracellular macromolecule permeability studies were performed (Figure 9.7). A2BR antagonism resulted in a significant loss of barrier function (increased macromolecule flux) compared to treatment with DMSO vehicle, NECA, and NECA combined with A1R antagonism (DPCPX). These results suggest that adenosine activation of A2BR induces filopodia that are functioning in cell-cell adhesions.

Figure 9.6. Electron photomicrographs of filopodia in HEC-1A endometrial carcinoma cells following NECA treatment and A2BR antagonism. HEC-1A cells were treated with 10 μ M NECA, 10 μ M NECA and 1 μ M MRS1754, or vehicle control (DMSO) and placed in hypoxia (1% O₂, 5% CO₂) for 36 hours. Changes in filopodia were assessed by transmission electron microscopy. (A) Representative electron photomicrographs of DMSO, NECA, and NECA and MRS1754 treated groups. Filopodia number increase with NECA treatment compared to DMSO. A2BR antagonism reduces the number of filopodia. Filopodia induced by NECA have variable length, which seems to be dependent, at least in part, on the distance to the nearest cell. (B) Representative electron photomicrograph of NECA treated cells and a higher magnification image showing interdigitating filopodia. Filopodia appear to be imbedding into the cytoplasm of a neighboring cell. Electron photomicrograph magnification (A) 5,000X and (B) 5,000X and 25,000X (magnified image)

Figure 9.6

A



B

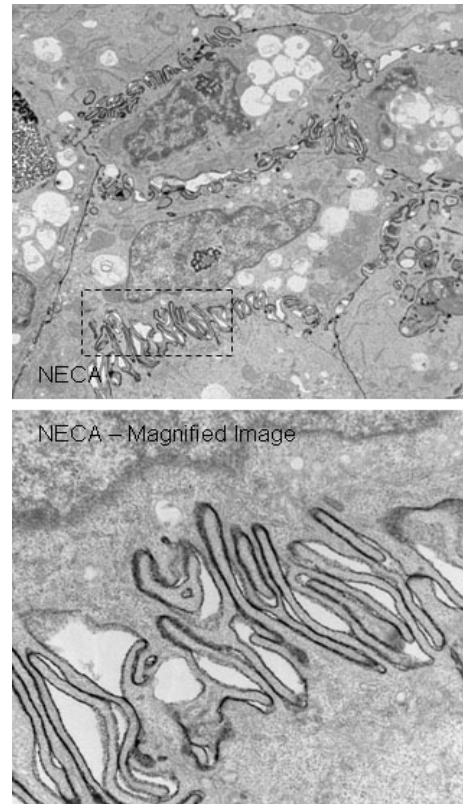
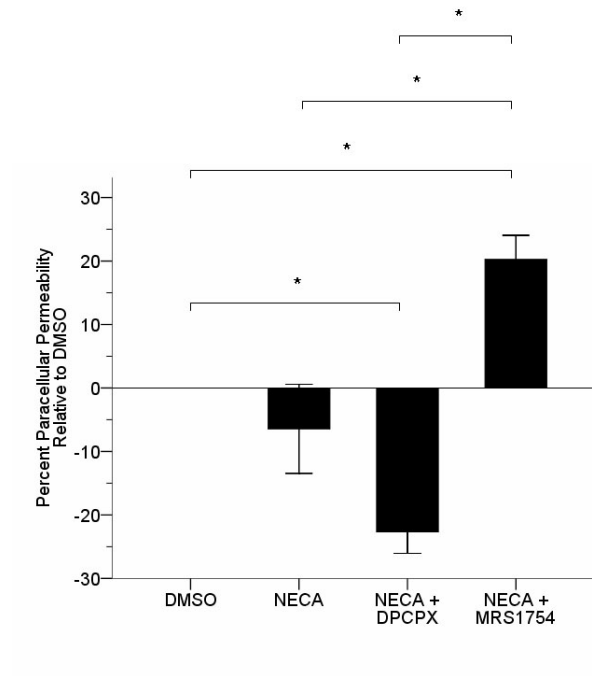


Figure 9.7. Macromolecule paracellular permeability in HEC-1A endometrial carcinoma cells following antagonism of A2BR and A1R. FITC-dextran (40kDa) paracellular permeability was measured following 36 hours of hypoxia (1% O₂, 5% CO₂). HEC-1A were treated with 10μM NECA, 10μM NECA and 1μM DPCPX 10μM NECA and 1μM MRS1754, or vehicle control (DMSO). Data are presented as percent paracellular permeability relative to DMSO. Values represent the mean ± SE of triplicate measurements from 3 different experiments. A2BR antagonism (MRS1754) results in significantly increased FITC-dextran paracellular permeability compared to treatment with NECA or NECA and DPCPX. (*p<0.05).

Figure 9.7

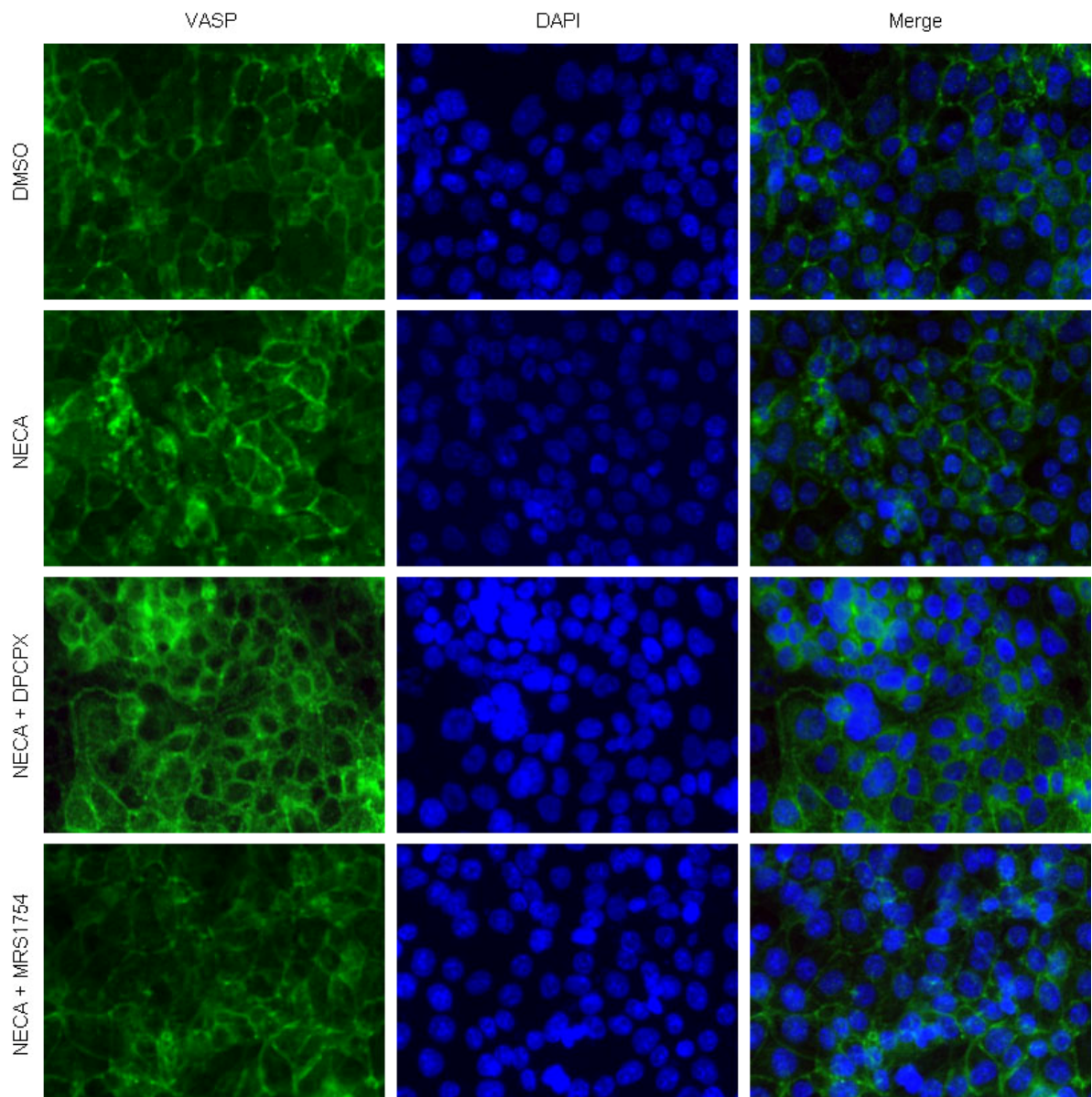


Adenosine's activation of A2BR increases the expression of membrane VASP in endometrial carcinoma cells, HEC-1A

Studies by Donald Lawrence and Katrina Comerford have described a NECA-induced, PKA-dependent, phosphorylation of VASP to be involved in moving VASP to tight junctions along with ZO-1 to provide adenosine-related barrier function in HMVEC and colon carcinoma cells, T84 (220, 221). VASP is known for its role in the formation of filopodia, but is also known to dually function in the stabilization of tight junction adhesions and assembly of the perijunctional actin-myosin II ring (220, 221, 303). The perijunctional ring is a thick circumferential belt composed of actin filaments. This ring is oriented parallel to the plasma membrane at the level of adherens junctions and is physically associated with this cell-cell adhesion structure. We hypothesized that increased expression of membrane VASP seen in these studies likely occurs in endometrial carcinoma cells via adenosine's activation of A2BR. In hypoxic (1% O₂, 5% CO₂) HEC-1A monolayers addition of NECA increased the membrane expression of VASP (Figure 9.7). However, antagonism of A1R (DPCPX) with NECA treatment greatly increased the membrane expression of VASP as compared to NECA alone. This is expected as NECA is a non-specific adenosine receptor agonist and activation of A1R negatively inhibits cAMP accumulation by A2BR. The increased expression membrane VASP is attributed to A2BR activation, as A2BR antagonism (MRS1754) resulted in decreased membrane VASP expression which is similar to that seen with the vehicle treated control, DMSO.

Figure 9.8. VASP expression in HEC-1A endometrial carcinoma cells following adenosine receptor antagonism. HEC-1A cells were treated with 10 μ M NECA, 10 μ M NECA and 1 μ M DPCPC, 10 μ M NECA and 1 μ M MRS1754, or vehicle control (DMSO) and placed in hypoxia (1% O₂, 5% CO₂) for 36 hours. An increased intensity in VASP membrane expression is seen with NECA treatment. With A1R antagonism (DPCPX) VASP membrane expression is increased beyond that of NECA. A2BR antagonism (MRS1754) results in decreased membrane VASP. Image magnification 20X.

Figure 9.8



Adenosine's activation of A2BR results in the cleavage of full-length vinculin to ~90kDa fragments in HEC -1A endometrial carcinoma cells.

Similar to VASP, vinculin is a dual resident of cell-matrix adhesions (focal adhesions) and cell-cell adhesions. At sites of cell-cell adhesion, vinculin functions to bind α -actinin (304), α -catenin (305, 306) or β -catenin (307) to provide increased stabilization of adherens structures to the actin cytoskeleton. Full-length vinculin exists in two conformations, a closed inactive conformation whereby its head domain interacts with its tail and an open active conformation (reviewed, (308)). Vinculin's active conformation is dependent on the binding of two different ligands (309, 310). Studies have recently suggested, however, that proteolytic cleavage of vinculin by calcium-dependent protease, calpain, may also function to activate vinculin (311). In these studies, vinculin fragments of ~95kDa and ~90kDa were found in the cortical cytoskeleton fraction of aggregating platelets and assumed to be important in actin remodeling (311). Interestingly, with platelet aggregation, platelets lose their characteristic shape and acquire extensive filopodia that function to help in the "clumping" (cell-cell contact) of platelets. Given our previous findings that adenosine activation of A2BR induces filopodia (actin remodeling) and is associated with membrane VASP expression and barrier function, we considered that vinculin could also be activated by adenosine's activation of A2BR. Vinculin expression was assessed in HEC-1A cells that were treated with the adenosine analog, NECA, NECA and the A2BR antagonist, MRS1754, or vehicle control, DMSO. NECA treatment caused full-length vinculin to be cleaved into a ~90kDa and ~95kDa fragment (Figure 9.9). As expected, the cleavage of full-length vinculin was more apparent in HEC-1A cells treated with NECA and the A1R antagonist (DPCPX) due to the non-specific agonist function of NECA. The ~90kDa fragment is related to A2BR activity, as indicated by its disappearance with antagonism of A2BR. These experiments show that adenosine's activation of A2BR results in the cleavage of full-length vinculin to a ~90kDa fragment. Studies indicate that proteolytic cleaved vinculin fragments interact more

efficiently with their protein binding partners than does full-length vinculin (reviewed, (308)). We therefore believe that the ~90kDa fragment may be acting one of two ways. First, it may be functioning to stabilize cell-cell adhesions to the actin cytoskeleton by binding with actin binding proteins, α -actinin and/or α -catenin. Second, it may be functioning to in the formation of cell-cell adhesion filopodia by its involvement in actin remodeling, as suggested in studies of aggregating platelets (311). To demonstrate that cleaved vinculin fragments are functioning either to increase cell-cell adhesion stabilization or the formation of cell-cell adhesion filopodia or both, HEC-1A cell migration following treatment with adenosine receptor antagonists in the presence or absence of anti-vinculin antibody was assessed. The anti-vinculin antibody used recognizes amino acid residues in the head region of vinculin, which involves regions within the ~95kDa and ~90kDa cleaved fragment. NECA and NECA and A1R antagonist (DPCPX) treatment significantly inhibit HEC-1A migration (Figure 9.10) Vinculin neutralization negated this effect, indicating that vinculin fragmentation is necessary for NECA-mediated inhibition of cell migration. In these experimental conditions, it is likely that adenosine activation of A2BR causes fragmentation of vinculin, and then vinculin's cleaved fragment either stabilizes newly formed or existing cell-cell adhesions and/or increases the induction of cell-cell adhesion filopodia. Further studies are needed to detail this association. However, regardless of function, both events are negative contributing effects to EC cell migration.

Figure 9.9. Vinculin immunoblot in HEC-1A endometrial carcinoma cells following treatment with NECA and adenosine receptor antagonists. HEC-1A cells were treated with 10 μ M NECA, 10 μ M NECA and 1 μ M DPCPC, 10 μ M NECA and 1 μ M MRS1754, or vehicle control (DMSO) and placed in hypoxia (1% O₂, 5% CO₂) for 36 hours. Full-length vinculin is 130kDa. Prominent ~90kDa (asterisk) and ~95kDa fragments of vinculin are seen with NECA treatment. With A1R antagonism a near complete loss of full-length vinculin along with the increased presence of the ~90kDa is found. The ~90kDa fragment is related to A2BR activity, as indicated by its disappearance with antagonism of A2BR.

Figure 9.9

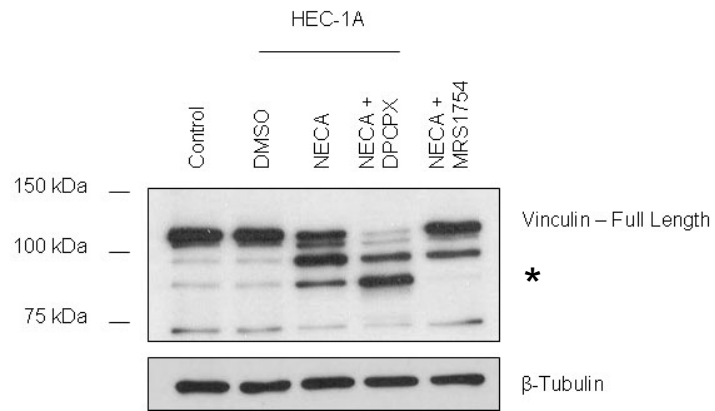
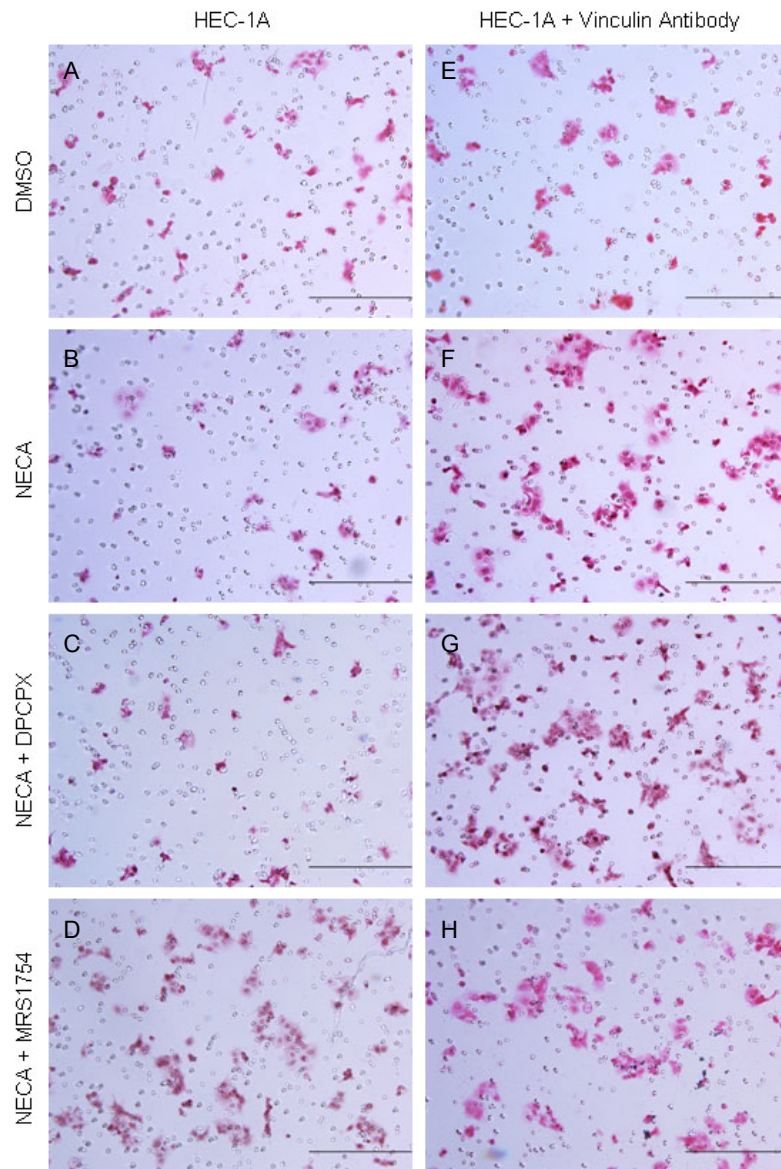


Figure 9.10. Representative images of HEC-1A endometrial carcinoma cell migration following adenosine receptor antagonism and anti-vinculin antibody treatment. HEC-1A cells were pre-treated with (E-H) or without (A-D) 10 μ m/ml anti-vinculin antibody and then treated with 10 μ M NECA, 10 μ M NECA + 1 μ M DPCPC, 10 μ M NECA + 1 μ M MRS1754, or vehicle control (DMSO). HEC-1A cells were subjected to hypoxia (1% O₂, 5% CO₂) for 24 hours. HEC-1A migration is reduced by (B) NECA and (C) NECA and DPCPX (A1R antagonism) compared to DMSO or NECA and MRS1754 (A2BR antagonism). The inhibitory effects of NECA and NECA and DPCPX are lost with pre-treatment with anti-vinculin antibody (F and G). Image magnification 10X.

Figure 9.10



Summary

The work presented within this specific aim has described a very novel discovery that adenosine's activation of A2BR induces filopodia that have cell-cell adhesion function. The function of cell-cell adhesion filopodia is well-described in embryonic development and with wound healing (223-225). Such an event is important for the proper alignment and development of mature adhesion structures. Importantly, we have functionally shown that adenosine's activation of A2BR increases barrier function under the identical conditions in which the filopodia induced by A2BR were described. Data here has shown A2BR to induce the expression of filopodia protein VASP and also demonstrated a novel relationship between A2BR activity and full-length vinculin cleavage. The ~90kDa fragment of vinculin has recently been suggested to a central protein involved in rescuing cell-cell adhesions by stabilizing membrane bound E-cadherin (312). Our migration studies involving the neutralization of vinculin functionally support that the ~90kDa fragment is inhibiting the progression of carcinoma cells.

CHAPTER TEN

Discussion

Specific Aims Overview

In the first specific aim, the initial findings of the purinergic pathway in relation to EC were introduced. These findings established the rationale for studying CD73 and adenosine's barrier function. In specific aim 2 the involvement of CD73-generated adenosine and A2BR in endometrial epithelial barrier function was mechanistically established. In specific aim 3, the expression of CD73 in normal and EC was characterized in detail. This specific aim also established that CD73 expression in carcinoma cells is significantly correlated to the epithelial phenotype. With specific aim 4, it was demonstrated that the mechanism of adenosine's barrier function, Stressor → CD73 → Adenosine → A2BR → Barrier Function, is the same mechanism that inhibits EC cell migration and invasion. Finally, in specific aim 5, a detailed molecular mechanism for adenosine regulation of cell-cell adhesion filopodia is provided.

CD73 is significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic endometrial carcinomas

Previous studies of CD73's expression in human cancers has not been conclusive and has not provided consistent results (breast (152, 313, 314); gastric (315); bladder (315); kidney, (316); glioblastoma (317); colon (314, 318, 319); thyroid (320); larynx (321)). Even among studies of a single cancer type, such as breast, there is much disagreement as to whether CD73 is down-regulated or over-expressed (breast (152, 313, 314)). A consensus of all studies would tend to suggest an over-expression of CD73 in cancer. However, data from this dissertation support the assumption that CD73 is down-regulated in a number of different carcinomas. This assumption is based on the following: 1.) our observations that *CD73's* expression is down-regulated not only in higher grade EC but also in ovarian high grade papillary serous carcinoma (HGpsc); 2.) banked

microarray data from publicly accessible databases, such as NextBio, demonstrate that *CD73* is significantly down-regulated in higher grade and/or advanced stages of endometrial, breast, prostate, ovarian, colon, adrenal, and kidney carcinomas; and 3.) previous studies present data using low sample numbers (n=15 or less (313-315, 320, 321) and/or do not take into account differences in expression by cancer grade or stage (314, 319). This third issue is especially important, as this has posed a great problem in trying to draw conclusions and understanding from the current literature. Established histological grading and staging parameters for EC, as with many cancer types, has shown to have prognostic importance (322). Importantly, when assessing expression of genes, these differences in grade and stage need to be accounted for, as they define distinct tumor biologies which occur with disease progression.

The need for separation by grade and stage is supported by the significant differences in *CD73* expression we observed in different EC grades and stages. This work has clearly shown that *CD73* is significantly down-regulated in moderately- and poorly-differentiated, invasive, and metastatic ECs. In specific aim 3, these findings were validated by double-labeling immunofluorescence, HPLC, and enzyme histochemistry. Specific localization of *CD73* expression and enzyme activity is especially important in cancer tissues, which are a complex mixture of carcinoma cells, desmoplastic stromal cells, inflammatory cells, and vasculature. From specific aim 3, double-labeling immunofluorescence and enzyme histochemistry showed *CD73* to be low to absent in carcinoma cells of poorly-differentiated ECs, but retained in carcinoma cells of well-differentiated ECs. Importantly, the primary source of *CD73* in poorly-differentiated ECs is the remaining surrounding stromal cells and connective tissue. In a large histochemistry study assessing *CD73* expression in 102 breast carcinomas of various disease stages, only 9 contained *CD73*-positive carcinoma cells (152). Close examination of this study reveals that the stromal cells are the primary cell type strongly positive for *CD73* (152). Prior studies using whole tissue homogenized assays of breast cancer have shown *CD73* to be over-expressed in carcinomas (313).

In aggregate, these data suggest that there is a stromal cell carryover effect that is explaining the high CD73 expression in whole breast cancer tissues. Such an effect of stromal cells is a significant problem of gene expression studies in breast cancer, as stromal cells are often the predominant cell type in a given unit area of a breast cancer. A unique feature of EC is that as the disease progresses, the stromal cells in the endometrium disappear (323). Therefore, the carryover gene expression from stromal cells is low which makes our observations more centered on the effects of CD73 in carcinoma cells rather than the surrounding stromal cells of the microenvironment.

CD73 expression being down-regulated in EC compared to normal endometrium is interesting when considering what is known about the purinergic pathway, its generation of adenosine, and disease. CD73 is the primary ecto-enzyme responsible for the generation of extracellular adenosine (157, 324). A consistent theme when considering adenosine's role in disease is that dysregulated expression of members of the purinergic pathway, including CD73 over-expression, serves to increase extracellular adenosine levels and therefore contributes to the adverse effects of the disease (236-239) (reviewed, (235)). Much of this understanding has been established in chronic lung diseases, including asthma, chronic obstructive pulmonary disease, and interstitial lung disease (reviewed, (235)). Elevated levels of adenosine in these diseases leads to tissue destructive inflammation, interstitial fibrosis, airway remodeling and hyperactivity, and mucus metaplasia (145, 168, 325-328) (reviewed, (235)). Importantly, these events occur because of the overactive and out-of-control production of adenosine. This increased production of adenosine initially is a protective response of damaged cells attempting to regain homeostasis. However, with time and with chronic stimuli, this tissue protective role of adenosine becomes tissue destructive. Given these well-established findings in chronic lung diseases, the question arises to why CD73-generated adenosine is down-regulated in EC. Aside from the reason that EC and chronic lung diseases are two very different diseases, a second more important reason of cell-

type specific biologies is likely the attributed cause. Extracellular adenosine has varying functions which are dependent on cell-type. In platelets adenosine is known to inhibit aggregation (329), but in endothelial cells it promotes both barrier function (202) and angiogenesis (330). In epithelial cells adenosine promotes barrier function (204), and in immune cells it can have both pro-inflammatory and anti-inflammatory effects (reviewed, (235)). The needs of carcinoma cells compared to other cells of the tumor microenvironment differ. It is very possible that while CD73 may be down-regulated in EC carcinoma cells, other cellular components of the microenvironment may retain CD73 expression, such as endothelial cells for the induction and support of angiogenesis. It is important to emphasize that while extracellular adenosine levels in mouse models of cancer have been measured at 0.2-2.4 μ M, mean 0.5 μ M (331) these levels are nowhere close to those seen in chronic diseases such as arthritis (332), asthma, and chronic obstructive pulmonary disease (168), which can typically have mean levels of extracellular adenosine of approximately 100 μ M. Basal extracellular adenosine levels in normal, non-diseased tissues has been estimated at 40-600nM (169). It has yet to be shown that extracellular adenosine levels in cancer patients or human carcinomas are at levels consistent with those considered to be chronic. It is possible that adenosine generated by the tumor microenvironment, including residential (stromal) or infiltrating (leukocytes) cells likely produces basal levels of adenosine that are sufficient to activate high affinity adenosine receptor subtypes (A1R, A2A, and A3R) that are over-expressed on carcinoma cells to provide to the progression of carcinoma. In colon carcinomas A3R over-expression has been indicated and shown to function to promote carcinoma cell proliferation (333). More studies are needed among all cancer types to really understand the significance of adenosine in cancer.

CD73-generated adenosine and A2BR induce endometrial epithelial barrier function: the three central themes to adenosine's barrier function effect.

As presented within the General Introduction, there are three events that can be emphasized as being central themes to adenosine's barrier function effect. One, adenosine's barrier function is a product of accumulated extracellular adenosine. Two, adenosine's barrier function is a product of CD73's generation of adenosine and its activation of A2BR (Stressor → AMP → CD73 → Adenosine → A2BR → Barrier Function). Three, adenosine's barrier function effect is supported by shifts in expression of purinergic pathway member.

Central Theme One and Two

Using previously characterized CD73^{-/-} mice (205) or A2BR^{-/-} mice (206) and by intraperitoneal injection of an adenosine analog, NECA into CD73^{-/-} mice we showed the relation of the first two themes to be relevant to inducing barrier function in endometrial epithelial cells. Importantly, as found in studies by Kristen Synnestyedt in intestinal epithelial cells (204) the barrier function induced by adenosine in normal epithelial endometrial cells was also found as a response to cellular stress. Barrier function among epithelial cells of the endometrium is the highest during the secretory phase of the endometrium. Estradiol significantly disrupts barrier function (334). Interestingly, in study that isolated normal epithelial cells of the endometrium from C57BL/6 mice, progesterone treatment of these endometrial epithelial cells did not increase transelectrical resistance – barrier function (334). This would suggest that genes induced by progesterone may not be involved with directly inducing barrier function. Our results demonstrated that CD73 is highly expressed in the secretory phase of the endometrium. However, progesterone was not found to induce CD73 expression.

Central Theme Three

Adenosine's barrier function effect is supported by changes in the expression of purinergic pathway members to support the extracellular accumulation of adenosine. This third central theme has been supported by previous studies demonstrating that cellular stress is associated with cytokine release or increased steady-state levels of HIF-1 α , which lead to the increased expression of members of the purinergic pathway important for the production (*CD39* and *CD73*) and activity (*A2BR*) of extracellular adenosine, while expression of members of the purinergic pathway that are important for the internalization of extracellular adenosine (ENTs) or its deamination (*ADA*) are concomitantly down-regulation. Overall, these events support the accumulation and activity of adenosine at the extracellular surface.

TGF- β 1

TGF- β 1 significantly induced *CD73* expression in EC cells via its Smad-mediated signaling pathway. *CD73* has only recently been described to be induced by TGF- β 1 in CD4+ and CD8+ T-cells, dendritic cells, and macrophages (335). These data reported here are the first to demonstrate a relationship between TGF- β 1 and *CD73* in non-immune cells. In intestinal epithelial cells, TGF- β 1 has been demonstrated to induce barrier function (188-193). It is interesting that a recent study has suggested the possibility of TGF- β 1's barrier function potentially being co-dependent on its induction of a cAMP-mediated barrier function (190). cAMP is a well-known second messenger of *A2BR*. The induction of *CD73* shown in specific aim 2 was associated with TGF- β 1's induction of barrier function in the EC cells. In our studies, we did not directly investigate whether the barrier function of TGF- β 1 and *CD73* are one in the same or two completely separate events. These issues can be addressed by pharmacological inhibitors of *CD73* and siRNA approaches, which were summarized in other parts of this dissertation. These studies are currently underway. An association between TGF- β and barrier function in the endometrium

has not been established at this time. An additional interesting consideration is whether TGF- β 1's induction of CD73 expression and barrier function may be one of the important, previously undescribed tumor suppressor functions of TGF- β in the early stages of carcinoma progression.

To speculate further on TGF- β 's involvement in the regulation of CD73 in the endometrium, it is interesting to emphasize the known expression of pattern of the TGF- β isoforms in the hormone active endometrium. Isoforms are increased in luminal and glandular epithelial cells during late proliferative phase and peak at early to mid secretory phase (336). CD73's expression has not been detailed for the entire menstrual cycle, but our work has clearly shown a significant difference, 2-fold increase, of CD73 expression in secretory endometrium. An important event has been TGF- β 's involvement with endometrial epithelial cell differentiation (reviewed, (337)). Data shown here has demonstrated that progesterone is not involved in increasing CD73 expression in the endometrium. The induction of CD73 in secretory endometrium may therefore be related to other secretory phase components, which may likely include TGF- β .

Increased Steady-State Levels of HIF-1 α

In hypoxia CD73 and A2BR expression levels are significantly up-regulated, CD73 6- and 20-fold (144, 204) and A2BR 12-fold (144). In EC cells, hypoxia did not increase CD73's (Specific Aim 2, Figure 6.7) expression with hypoxia despite HIF-1 α stability. Moreover, the induction of A2BR by hypoxia was significant however much lower, 1- and 2-fold, than expected. A few possible reasons may explain these results. First, these EC cells may have lost the cellular machinery necessary to mount a compensatory response to the induction of CD73 in hypoxia. Second, these EC cells may have a peak tolerance for how much CD73 they are willing to express. The compensatory response or mechanisms can be supported based on studies by Kristen Synnedyed, which assessed HIF-1's induced expression of CD73 by using luciferase reporter

promoter constructs of different lengths (204). Studies showed CD73's full-length promoter to be partially repressed in hypoxia as compared to its half-length promoter (204). Transcription factors GATA-1(338) and GATA-2 (339) have been implied to repress genes in hypoxia, and have consensus sites in the distal full-length promoter of CD73 (340). Thus, despite HIF-1 α 's presence CD73's expression could be negatively regulated by GATAs. Hypoxia is a characteristic feature of most carcinomas, including EC (269-272). Transcriptional events induced by HIF-1 increase the expression of genes that provide to carcinoma cell migration and invasion (341). It is reasonable that EC cells would induce mechanisms that would prevent HIF-1's induction of *CD73* given that increased expression of *CD73* induces barrier function. *A2BR* expression was also not robustly induced by hypoxia as expected in EC cells. This indicated that repressors of *A2BR* could also be involved in preventing a barrier function response. In Appendix B, we show that *de novo* methylation of CpG dinucleotides in *CD73*'s promoter does not appear to be involved in this situation. However, this epigenetic event may be partially involved with *A2BR*'s ability to be induced by HIF-1. Using Methylator SVM based software for DNA methylation prediction, courtesy of Harvard Bioinformatics, we identified two predicted methylated cytosines of CpG dinucleotides to fall directly within two HIF-1 binding motifs (5'-A/GCGTG-3') of *A2BR*'s promoter. Treatment of HEC-1A cells and WISH-HeLa cells with DNA demethylating agent 5-Aza-2'-deoxycytidine increased *A2BR* transcripts in hypoxia by 3-fold (HEC-1A) and 8-fold (WISH-HeLa) (Appendix, Figure 14.2). Further studies are needed to specifically define if methylation of *A2BR*'s promoter at HIF-1 binding motifs is a direct effect.

Functional Studies: Stressor → AMP → CD73 → Adenosine → A2BR → Inhibits Endometrial Carcinoma Migration and Invasion

Very few studies have addressed CD73 and/or adenosine in relation to carcinoma cell migration and invasion (breast (342-344)). Functional studies concerning CD73, adenosine, and

its receptor subtypes, such as A2AR and A3R, has involved assessing carcinoma cell proliferation ((thyroid (345) and colon (333, 346, 347)) or carcinoma cell avoidance of immunosurveillance (331, 348, 349). With all these studies, the use of *in vivo* and/or *in vitro* models that closely mimic the conditions that are seen in the primary cancer or *in vivo* microenvironment is of great importance. Previous migration and invasion studies assessing CD73 in breast carcinoma cells have not taken this into consideration. Studies have involved exogenously over-expressing CD73 or used supra-physiological concentrations (100µM) of adenosine (306, 308). These experimental conditions do not mirror the known *in vivo* expression for CD73 in breast carcinomas, as CD73 is reported to be nearly entirely absent from carcinoma cells in this cancer type (152). Therefore, it is difficult to draw hypotheses or conclusions from this work. The study design and EC cells used in our migration and invasion studies were characterized and selected based on our *in vivo* observations (CD73's induction of barrier function and CD73's down-regulation in carcinoma cells). It was also taken into consideration that standard tissue culture conditions, ambient oxygen content, are distinct from the conditions experienced by carcinoma cells *in vivo*. In fact, it is established that ECs are hypoxic *in vivo* (269-272). From our data, it was concluded that CD73, its generation of adenosine, and adenosine's activation of A2BR inhibit EC cell migration and invasion in a similar manner (Stressor → CD73 → Adenosine → A2BR) to that seen with adenosine's induction of barrier function. Barrier function and migration and invasion are mutually exclusive cellular events. Importantly, our results clearly show that down-regulation of CD73 in EC cells is not a passive event.

CD73 is best known and characterized for its function to produce adenosine from the phosphohydrolysis of 5'AMP (157). Studies in this dissertation focused on the catalytic activity of CD73 in normal endometrium and ECs. CD73 functions that are non-related to its catalytic activity include its cell-matrix interaction with ECM proteins, laminin, fibronectin (163, 164), and tenascin C (165). These functions have not been well-established and/or demonstrated in

mammalian cells. We can not discount the potential of CD73 having non-catalytic function in epithelial cells of the endometrium.

Adenosine's Activation of A2BR Induces Filopodia Zippering

Membrane Expression

A gap in knowledge in the adenosine field concerning adenosine's barrier function has been the lack of understanding of the events or proteins which A2BR regulates. A very unique observation was made in specific aim 5 concerning the restricted expression of CD73 to membranes of cell-cell contact. CD73 is a GPI-linked ecto-enzyme. GPI linked proteins are typically known to be targeted to the apical membrane of cells, while small amounts may localize to the basolateral membrane (350). The GPI linkage functions as an apical targeting signal (351-353) Our studies cryosections of normal endometrium demonstrated a greater level of expression of CD73 on the apical membrane of glandular epithelial cells with a lower level of expression found on the basolateral membrane (Specific Aim 3, Figure 7.1 C). These results are consistent to those described for colonic crypt epithelial cells (275). Studies assessing CD73 trafficking in polarized versus non-polarized hepatoma cells have shown that regardless of the 2D or 3D cultured state of cells, CD73's expression was dispersed among all membrane surfaces (301). Basolateral membrane localized CD73 has been demonstrated to be GPI-linked, as basolateral expressed CD73 was released from the membrane by phosphatidylinositol-specific phospholipase C (PI-PLC) (275). It is assumed that EC cell expressed CD73 is GPI linked. GPI-linked proteins are localized in specialized membrane domains known as lipid rafts (reviewed, (350)). Characteristics of lipid rafts, such as sphingolipid, ceramide, and/or cholesterol composition, are known to change in carcinoma cells, which provides benefit to the progression of the cells (reviewed, (354)). For example, cholesterol depletion causes the loss of EGFR in lipid rafts, which promotes its binding to EGF and phosphorylation (355). It is unknown if changes in lipid raft

dynamics are involved in the loss of CD73 expression on membranes not involved in cell-cell contacts. Lipid raft composition is different among lipid rafts that are at the apical membrane versus those that are basolateral and involved in cell-cell adhesions (reviewed, (354). Immunofluorescence studies involving newly forming cell-cell adhesions have described E-cadherin, β -catenin, and α -catenin to be located in lipid rafts.

Filopodia

Electron photomicrographs of EC cells showed finger-like projections (filopodia) among the paracellular spaces. These filopodia expressed CD73 and was catalytically active. Filopodia have a diverse array of functions, including participating in wound healing, adhesion to the extracellular matrix (focal adhesion), guidance towards chemoattractants, neuronal growth-cone pathfinding, and embryonic development (reviewed, (302)). For determining specific functions of filopodia, certain characteristics of the filopodia have to be taken into consideration. These characteristics include the following: 1.) the presence of a particular cell-matrix or cell-cell adhesion protein within the filopodia (focal adhesions - integrins; cell-cell adhesions - E-cadherin); 2.) the presence of certain structural or signaling proteins within the filopodia; and 3.) the presence of proteins that alter both the filopodia biochemical and biophysical features. In addition, the function of filopodia is dependent on cell type (reviewed, (298)). In EC cells filopodia extending between cells had the characteristic cell-cell adhesion feature of expressing E-cadherin along with filopodia protein VASP. Similar co-expression of E-cadherin and VASP are seen in the cell-cell adhesion filopodia seen in keratinocytes. (299). An important feature found in EC cells was the presence of inter-digitating filopodia, known a important feature found in EC cells was the presence of inter-digitating filopodia, known as adhesion zippers. Inter-digitation is a signature characteristic of cell-cell adhesion filopodia. Inter-digitation of filopodia was first identified to be important to dorsal closure and epithelial sheet sealing in *Drosophila*

melanogaster and *Caenorhabditis elegans* embryos (223-225, 299). The evolutionary conservation of this process highlights its importance in establishing and maintaining cell-cell adhesions. As seen with E-cadherin, CD73 was expressed in filopodia functioning to initiate cell-cell adhesions. It is unknown if CD73's presence in filopodia is essential for the direct formation of filopodia or if it is involved with latter events, such as adhesion zippering. The reduction in numbers of filopodia with A2BR antagonism strongly supports that adenosine activity is an important regulator of these filopodia. Filopodia that fail to form adhesion zippering will retract. When viewing filopodia among treatment groups it cannot be ruled out that lower numbers of filopodia in A2BR antagonized EC cells are due to a deficiency in adhesion zippering. Therefore, it is difficult to decipher at this time if adenosine induces filopodia or is directly involved in the adhesion zippering process. cAMP (356-358) and Ca^{2+} (299, 359) are both ubiquitous second messengers for many G protein-coupled receptors, including A2BR, and have been shown to induce filopodia.

Formation of Cell-Cell Adhesions

The overall importance of adenosine to induce cell-cell adhesion filopodia may be emphasized by the interesting epithelial-like phenotype of the EC cells, HEC-1B. In the initial formation of cell-cell adhesions, E-cadherin acts as the initiating event for cell-cell adhesion formation. By forming homotypic interactions with a neighboring cell's-expressed E-cadherin initiates the further development of the adherens junction and initiates cell polarization and tight junction formation (reviewed, (360)). It has been shown that in the event of E-cadherin deficiency or inactivation, the entire process of forming cell-cell adhesions can continue if cells are stimulated by cAMP, PKA, or, PKC (218, 361). cAMP, PKA and PKC are known to be important primary down-stream components of A2BR signaling (179). Moreover, stimulation of cells by PKA prevents the disassembly of tight junction structures in the presence of E-cadherin

inactivation (361, 362). HEC-1B cells, which have classic epithelial-like characteristics, lack E-cadherin expression, but they express twice the amount of CD73 than other epithelial-like EC cells, such as HEC-1A (Specific Aim 3, Table 7.2). Additionally, HEC-1B cells display very prominent cell-cell adhesion filopodia, as shown by immunofluorescence (Specific Aim 5, Figure 8.3). Taken together, these results suggest that CD73-generated adenosine activating A2BR is at least comparable in importance as E-cadherin is in maintaining and forming cell-cell adhesions. Importantly, this would also suggest that carcinoma cells that have lost E-cadherin expression and have thus become resistant to therapies may be successfully targeted to re-establish cell-cell adhesions by pharmacological agonists to A2BR.

Adenosine → A2BR → Filopodia → Vinculin → Adhesion Formation and Stabilization

cAMP induces filopodia formation on neurite shafts in a PKA-VASP phosphorylation dependent manner (356). Our studies here have utilized VASP for its nature of being restricted to certain types of filopodia, such as cell-cell adhesions versus focal adhesions. However, VASP is also a protein found among cell-cell adhesion structures. A PKA-dependent VASP phosphorylation event has been described for adenosine-mediated barrier function (endothelial cells (220)) (epithelial (221)). In these studies, VASP phosphorylation at the membrane (tight junction region) of HUVEC (220) and T84 (221) cells leads to the structural relaxation of the actin cytoskeleton. Our VASP immunofluorescence studies found increased total VASP at the membrane with adenosine treatment. However, from this work, it is difficult to define how much VASP may be related to filopodia and how much VASP is related to tight junction stabilization. It is unknown whether both events occur simultaneously or whether the amount of VASP for either function is dependent on the severity of the loss of cell-cell adhesions. Much work is needed in this area to further understand the biology of adenosine-induced VASP.

As with VASP, vinculin is expressed in both filopodia and cell-cell adhesions (307). Vinculin is as an important stabilizer of E-cadherin and β -catenin adhesions (307). Vinculin is divided into a head-domain (residues 1-835) and a tail-domain (residues 836-896). Vinculin has binding sites for many interacting proteins, including talin, α -actinin, α -catenin and β -catenin, Arp2/3, VASP, and actin (reviewed, (363)) Vinculin is commonly found in its inactive conformation, where it inhibits itself by binding its head-domain with its tail-domain (364, 365). It has generally been accepted that vinculin is activated by conformational change. This change occurs by the binding of actin to the tail-domain and talin to the head-domain (310). An additional mechanism of activation has included the interaction of phosphatidylinositol-4,5-bisphosphate (PIP₂) to the tail-domain (366, 367). It has recently been challenged that protease cleavage of vinculin may be an alternative activation event of vinculin (311). In platelets stimulated to aggregate, cleavage of ~90 and ~95kDa head-domain fragments from full-length vinculin occurs by calpain, a Ca²⁺ protease (311). An interesting event is that with platelet aggregation, platelets lose their characteristic disc shape and acquire extensive filopodia that function to “clump” the platelets together (cell-cell contact). Studies of recombinant vinculin or protease cleaved vinculin show vinculin to bind with higher efficiency to its binding partners than seen with full-length vinculin (304). The controversy with vinculin fragmentation has been the general concept that fragmentation results in the loss of the actin binding site of vinculin. For vinculin to function in stabilization of E-cadherin, it is thought that it must bind with α -catenin (head-domain) and actin (tail-domain) simultaneously. This currently accepted model is being challenged. In studies conducted by Madhavi Maddugoda, a recombinant head-domain of vinculin fused with α -catenin was shown to rescue the disruption of cell-cell adhesions by stabilizing membrane bound E-cadherin. Recombinant proteins of α -catenin alone or α -catenin fused with vinculin’s tail-domain were unable to rescue the loss of cell-cell adhesions (312). This work argues that fragmented vinculin functionally serves a role in the stabilization of cell-cell

adhesions (312). Concerning our work, it is unclear if the cleaved head-domain fragments of vinculin function in stabilization of cell-cell adhesions by binding α - and/or β -catenin with greater affinity, or if the vinculin fragments are functioning to induce filopodia. Currently, co-immunoprecipitation studies are underway to determine whether the cleaved ~90kDa fragment of vinculin has a great amount of bound α - and/or β -catenin. Our work has shown that the A2BR inhibited migration of EC cells is lost with neutralization of vinculin. With the cleavage of vinculin and its fragments having functional activity not being an event that is widely known and accepted at this time, additional studies will be needed to confirm the functionality of the ~90kDa fragment in EC cells.

CHAPTER ELEVEN

Conclusion

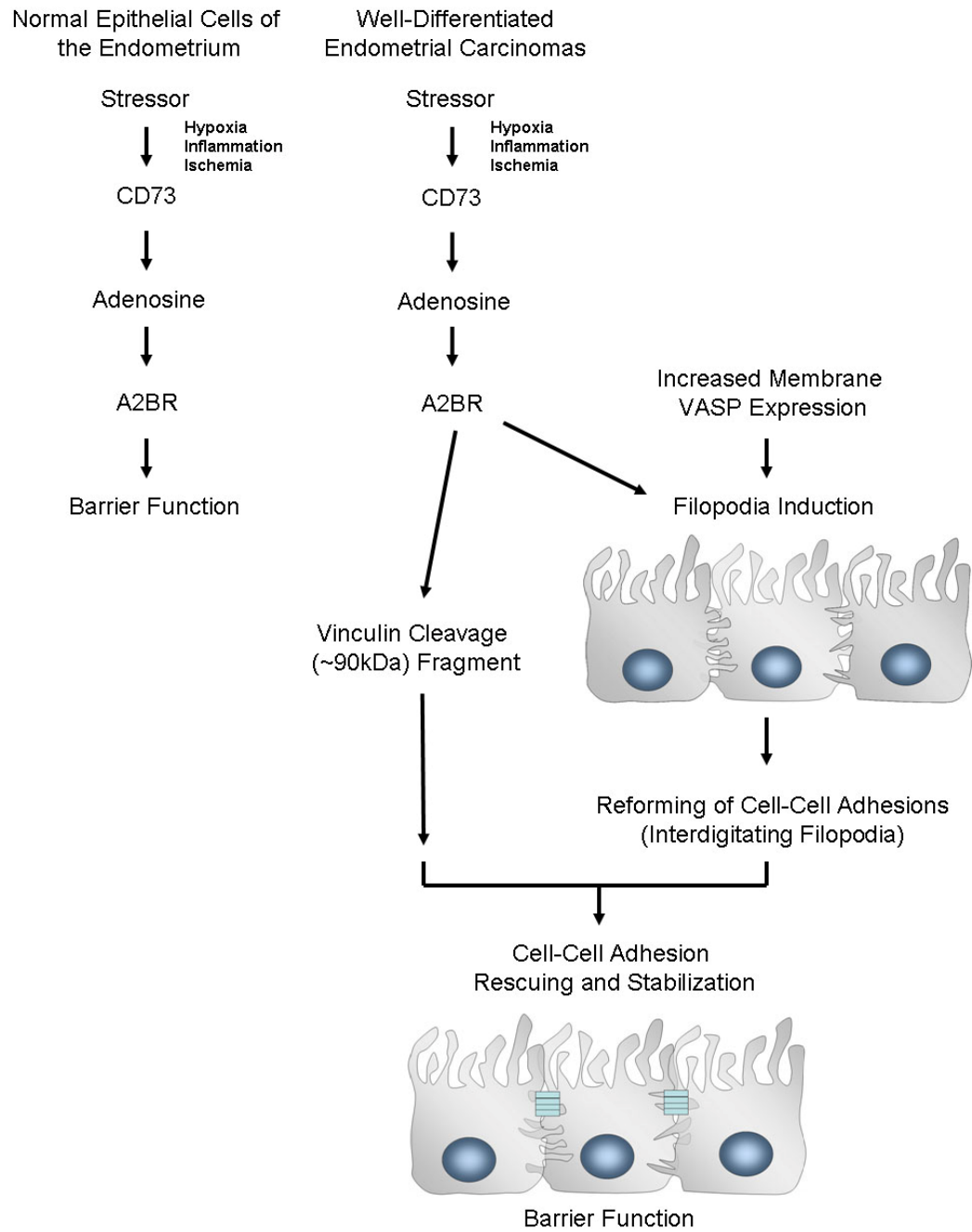
The work in this dissertation has established the basic science and clear mechanistic association between adenosine and its regulation of cell-cell adhesions in carcinoma cells. CD73 was shown to be significantly down-regulated in carcinoma cells of moderately- and poorly-differentiated, invasive, and metastatic ECs, and that the primary down-regulation of CD73 in ECs were in the carcinoma cells. Importantly, we showed that CD73-generated adenosine and its activation of A2BR was involved in inducing barrier function, a homeostatic response, in normal epithelial cells of the endometrium. In EC carcinoma cells, the loss of CD73 in well-differentiated EC cells increased migration and invasion via Stressor \rightarrow CD73 \rightarrow Adenosine \rightarrow A2BR. This is the identical cascade of CD73's induction of barrier function. Moreover, adenosine-induced filopodia by A2BR activity was found to be involved in reforming cell-cell adhesions in EC carcinoma cells. From this work, we conclude that it is necessary for EC cells to down-regulate CD73 in order to avoid the barrier function effects from adenosine's activation of A2BR. More importantly, the down-regulation of CD73 and therefore generation of extracellular adenosine is important to preventing the reformation of cell-cell adhesions by adenosine-induced filopodia. Loss of cell-cell adhesions provide not only to carcinoma cell migration and invasion, but also provide to carcinoma cell proliferation and basolateral receptor activity. These two events are important for the early progression of carcinomas. Mechanisms involved in the loss of cell-cell adhesions have been well-described. What remains understudied and something that is not readily considered are the mechanisms and molecular components responsible for reforming cell-cell adhesions once they have been weakened or lost in carcinoma cells. Importantly, it is known that carcinoma cells of early stage cancers are not too far separated from the genetic and molecular profile of normal epithelial cells. Epithelial cells have an inherent nature to maintain contact with neighboring cells. This would suggest that molecular mechanisms that regulate the recognition

and maintenance of cell-cell contact would be important targets to be down-regulated in carcinoma cells, especially in early stage disease. In this work, CD73 was found to be down-regulated relatively early in EC. Therefore, it is quite possible that CD73-generated adenosine and adenosine's induction of cell-cell adhesion filopodia-induced zippering is one of the homeostatic mechanisms that responds to reforming cell-cell adhesions under cellular stress. This is truly a novel finding that has potential for providing therapeutic benefit. The therapeutic potential of this work is discussed in Chapter 13. A schematic that highlights the central data presented in this dissertation and the conclusions that have been made are detailed in Figure 11.1.

Figure 11.1. Summary schematic. (A) CD73 expressed in normal endometrial epithelial cells generates adenosine in response to cellular stress. This extracellular generated adenosine activates A2BR to induce barrier function. In well-differentiated EC cells that retain CD73 expression, adenosine generated by CD73 activates A2BR and induces filopodia formation. Proteins that support the formation and function of filopodia, such as VASP, are induced by A2BR activity. Filopodia of these well-differentiated EC cells function to reform cell-cell adhesions by interdigitating among one another. Activity of A2BR also results in the cleavage of full-length vinculin, producing a ~90kDa fragment. The combination of filopodia inducing the reforming of cell-cell adhesions and ~90kDa vinculin acting to rescue cell-cell adhesions significantly inhibits carcinoma cells from de-attaching from their neighbors. Therefore, the homeostatic event, barrier function, remains intact in these carcinoma cells, which significantly inhibits the progression of these carcinoma cells. (B) In poorly-differentiated ECs, CD73's down-regulation in carcinoma cells significantly promotes the migration and invasion of these carcinoma cells, as the carcinoma cells are unable to produce extracellular adenosine.

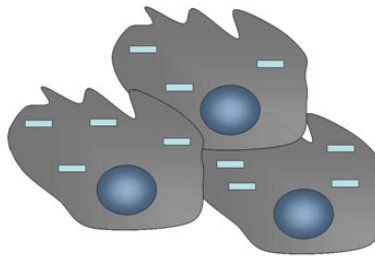
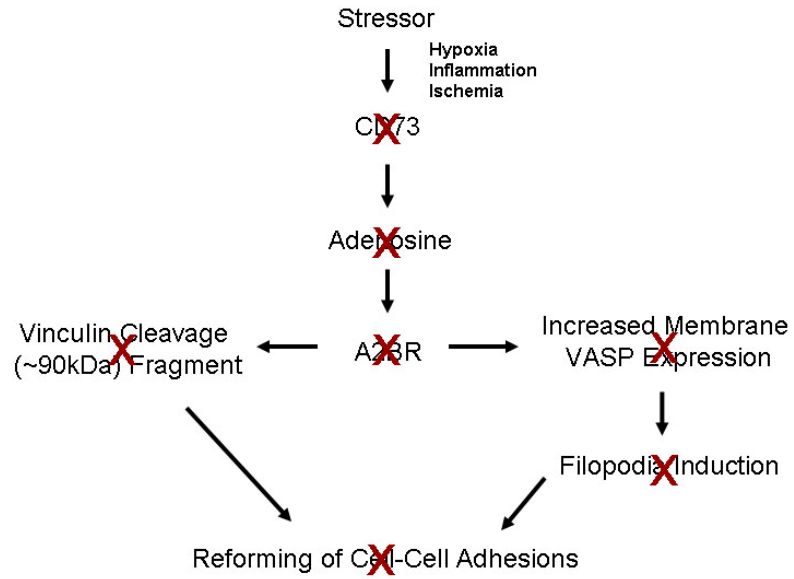
Figure 11.1

A



B

Poorly-Differentiated
Endometrial Carcinomas



Migration and Invasion

CHAPTER TWELVE

Future Directions

Development of Mouse Models of Invasive Endometrial Cancer

The *PTEN*^{+/-} mouse model typically develops endometrial hyperplasia and, in a smaller subset of mice, endometrial carcinoma. Onset of hyperplasia is much delayed, reliably appearing when the mice are 6 months of age. Cancers take longer to develop (368). It would be interesting to cross the *PTEN*^{+/-} mice with *A2BR*^{-/-} and *CD73*^{-/-} mice. Given the important role of CD73 activation of A2BR in forming cell-cell contacts, we would hypothesize that the resulting mice might have more invasive cancers at a younger age (acceleration of the phenotype). Recently, homozygous *PTEN* deletion has been successfully targeted to the uterus. These mice all develop EC within 1 month of age with myometrial invasion by 3 months of age (369). This would be an ideal model to try therapeutic approaches, such as use of an A2BR agonist, that would promote adenosine-signaling and promotion of cell-cell contacts.

Adenosine's Association with PTEN via Vinculin

PTEN is one of the most frequently inactivated tumor suppressors in cancer (370). PTEN loss is especially important in EC, as approximately 80% of endometrioid ECs and 55% of premalignant endometrial lesions have loss of PTEN (18). PTEN loss is functionally important, as *PTEN*^{+/-} mice develop endometrial hyperplasia with high penetrance (approximately 100%) and endometrial carcinoma (approximately 25% (371)). PTEN acts as a tumor suppressor by negatively controlling the PI3K/Akt signaling pathway, an important pathway for cancer cell proliferation and survival. Inactivation of PTEN in EC occurs either by mutation, deletions, promoter hypermethylation, or loss of protein function (370). Recently, vinculin has been shown to control PTEN protein levels by maintaining the interaction of β -catenin with the membrane scaffolding protein, membrane-associated guanylate-kinase inverted 2 (MAGI-2). In this

dissertation, it was shown that adenosine's activation of A2BR regulates the cleavage of vinculin (Specific Aim 5, Figure 9.9). These fragments are currently being assessed for increased binding efficiencies to α -catenin and β -catenin. Future studies will evaluate the hypothesis that adenosine activation of A2BR stabilizes PTEN protein via cleavage and activation of vinculin.

CHAPTER THIRTEEN

Translational

Once a cancer has metastasized there is no definitive cure, and in many cases clinical focus shifts from cure to slowing the spread of the disease. Unfortunately, many individuals diagnosed with cancer have dissemination of the disease at the initial time of its discovery. Therapy resistance remains a significant clinical issue with metastatic disease. Currently, there are no mechanisms that can re-sensitize cancer cells to therapy. Sensitivity to chemotherapy agents is associated with the presence of individual cell-cell adhesion proteins in carcinoma cells. These proteins include E-cadherin (110-115), β -catenin (113, 115), α -catenin (110), γ -catenin (110, 113, 114, 116), and claudin-7 (110). Importantly, these laboratory-based observations are clinically relevant (110), as re-expression of cell-cell adhesion proteins in carcinoma cells in the experimental setting has the potential to improve or re-sensitize these cells to therapeutic agents. A great challenge, however, has been the current inability of these proteins to be drug targeted in the clinical setting. Our interests with this dissertation work has included the relevance of adenosine's ability to induce cell-cell adhesions and this potential scheme being of clinical usefulness in improving sensitivity or re-sensitizing carcinoma cells to current therapy. As shown in this dissertation, adenosine's activation of A2BR induces filopodia-driven cell-cell adhesions in EC cells. A2BR activity is closely associated to the secondary messenger cAMP, and cAMP has been indicated in many studies to be involved in filopodia formation. The relevance of cAMP activation is important as it is shown that cell-cell adhesion formation can proceed even in the presence of E-cadherin deficiency (218, 361).

In this chapter, a clinical introduction of EC is given. This includes information on the current standard of care for EC, clinical trials that are on-going in this disease, and a brief overview of some of the current areas of clinical and translational interest in the management of

EC. The remainder of the chapter will discuss the potential applications of this dissertation's work to the clinic.

Clinical Introduction to Endometrial Cancer

Endometrial cancer (EC) is the fourth most common cancer diagnosis among women in the United States. In 2011, 46,470 women are expected to be newly diagnosed with EC. With an estimated mortality of 8,120 women for 2011, EC ranks eighth for cancer-related deaths (1). The incidence of EC over the past 10 years has continued to increase at a concerning rate which is attributed to increased life expectancy and obesity (3, 4).

Most ECs are of the endometrioid histology, followed by uterine papillary serous and clear cell carcinomas (6). EC is a disease that is divided into two distinct categories, Type I and Type II, based on clinicopathologic and molecular data. Type I ECs (approximately 80% of EC diagnoses) comprise the endometrioid carcinomas that are often preceded by atypical hyperplasia and express the estrogen receptor (ER) and progesterone receptor (PR) (372). Type I ECs are low grade and rarely metastasize (373). If diagnosed at an early stage, the prognosis of Type I ECs is favorable, with 5-year survival rates of 97% for FIGO, stage I and 80% for FIGO stage II (373). Type II ECs (approximately 20% of EC diagnoses) comprise non-endometrioid subtypes, including uterine papillary serous and clear cell carcinoma. Type II ECs are high grade and arise in the background of an atrophic endometrium and appear to be un-related to high estrogen levels (7, 374). Type II ECs are highly aggressive clinically and, despite their lower prevalence, account for a large proportion of EC-related mortalities (45-50%) (375). More than 60% of women diagnosed with Type II EC have metastasis at the time of diagnosis (7). The 5-year survival rates for FIGO stage III disease (regional metastasis) and stage IV disease (distant metastasis) is 43% and 3%, respectively (376). When combined as a whole (Type I and Type II), the majority of

ECs (72%) are diagnosed in early stages, FIGO stage I or II, whereas 20% will have regional metastasis (FIGO stage III) and 8% distant metastasis (FIGO stage IV) (38).

The epidemiology of endometrial cancer is multifactorial. Most cases are sporadic and develop in postmenopausal women. The median age of diagnosis is 61 years (6). Common risk factors associated with Type I ECs include unopposed estrogen exposure and obesity (377). Unopposed estrogen is most related to estrogen replacement therapy or tamoxifen use, reproductive issues causing anovulation, and obesity. (6, 378). Smaller subsets of sporadic ECs, those that are related to Type II disease, are associated with aging and the unique genetic and molecular changes that occur with these more aggressive variants (39). Approximately 2-5% of ECs are associated with a hereditary syndrome, hereditary nonpolyposis colorectal cancer syndrome (HNPCC), also known as Lynch syndrome. HNPCC is caused by germline mutations in DNA mismatch repair genes, *MLH1*, *MSH2*, *MSH6*, and *PMS2* (379).

Unlike many cancers, EC presents with a distinct symptom, abnormal uterine bleeding, which occurs in 90% of patients. An EC diagnosis occurs in ~10% of patients with postmenopausal bleeding (PMB). In 20-40% of patients, PMB is caused by endometrial hyperplasia, endometrial polyps, or a miscellaneous benign pathology of the endometrium. In the remaining 50-70% of patients, PMB is often attributed to endometrial or vaginal atrophy (380). Screening methods are not typically implemented for EC. Papanicolaou (Pap) smears are a very poor screening test for EC (381), and screening for EC using transvaginal ultrasonography in asymptomatic postmenopausal women has a poor positive predictive value (9%) and is not recommended (382).

Management of Endometrial Cancer – Standard of Care

For EC, the mainstay therapy is surgery and includes a total hysterectomy (removal of uterus and cervix), bilateral salphingo-oophorectomy (removal of ovaries), and assessment of

peritoneal cytology. For women with non-endometrioid carcinomas, deeply invasive low grade endometrioid carcinomas, and many grade 3 endometrioid endometrial carcinomas, additional procedures are added to this surgical approach, including removal of the omentum, bilateral pelvic lymphadenectomy, para-aortic lymphadenectomy, and peritoneal biopsies, including biopsies of the bladder, diaphragm, right/left colic gutters, right/left pelvic sidewalls, small- and large-bowel serosa and mesentery, and other suspicious tissues (383). Survival and the use of adjuvant therapies are greatly dependent on surgical stage.

Adjuvant therapies for EC include radiation, anti-hormone therapy, and single-agent and combination chemotherapy. External pelvic radiotherapy and/or vaginal brachytherapy is used postoperatively for patients at high risk for local recurrence, such as those having a grade 3 endometrioid carcinoma or a stage IC tumor and those with poor prognosis (384). External beam radiotherapy and/or intrauterine brachytherapy is also used as a primary therapy for medically inoperable patients (385, 386). Historically, chemotherapy was reserved to treat patients with metastatic or recurrent disease following failure of hormone therapy (39). As a result of recent Phase II and III clinical trials, chemotherapy is now standard of care for patients with locally advanced or metastatic disease. Doxorubicin, paclitaxel, and platinum agents (cisplatin and carboplatin) are the most active chemotherapy agents in EC. In chemotherapy-naïve EC patients, single-agent response rates are 17-37% for doxorubicin, 36% for paclitaxel, 20-42% for cisplatin, and 24-33% for carboplatin (reviewed, (39, 387). Phase III clinical trials have subsequently compared the efficacy of combination chemotherapy in EC, including the comparison of doxorubicin-cisplatin versus doxorubicin-paclitaxel-cisplatin (388). Combination chemotherapy regimens, including the three agent combination of doxorubicin-paclitaxel-cisplatin, have shown significantly greater response rate (RR), progression free survival (PFS), and overall survival (OS). Interestingly, these increases have only been incremental (approximately 2 months). Current standard of care for EC generally includes combination chemotherapy regimens versus

that of a single-agent alone. Though combination regimens are the most active, responses are observed in only 50% of patients, and a complete response is infrequently observed. PFS and OS have improved, but the 5-year survival rate for patients with advanced or recurrent EC has remained unchanged (reviewed, (39)). Promising second-lines of treatment are limited. Second-line chemotherapy is limited to response rates of 4% for cisplatin, 7.7% for docetaxel, 12% for ixabepilone, 13.5% for oxaliplatin, 15% for ifosfamide, and 27.3% for paclitaxel (reviewed, (39)) (389). Hormonal therapy, primarily progestins, is less toxic than chemotherapy, and has a 20% RR in patients with PR+ EC. Hormone therapy use is also common in elderly patients because of its low toxicity level, in younger women wishing to maintain fertility, and medically inoperable patients (reviewed, (3)). Targeted therapies have yet to be introduced into routine clinical practice, although many are the subject of early phase clinical trials (reviewed, (390)).

Targeted Therapy

A major advance in the treatment of cancer over the past decade has been the introduction of agents that target specific molecular abnormalities in cancer cells. Common examples include trastuzumab, a monoclonal antibody against Her2/neu used in Her2/neu-positive breast cancer; rituximab, an anti-CD20 monoclonal antibody used in non-Hodgkin's lymphoma; imatinib, a tyrosine kinase inhibitor used in KIT-positive gastrointestinal sarcomas; and sunitinib, a tyrosine kinase inhibitor used in renal cell cancer. The attractiveness of targeted agents is their advantage of being more selective against cancer cells than healthy cells. Conventional chemotherapy is non-selective in that it targets fast growing/dividing cells, which may or may not be cancer cells. Because of this, toxicity is a significant issue with chemotherapy. Along with being more specific, targeted agents have proven to be less toxic and, in some cases, have improved efficacy over conventional chemotherapy. Phase III clinical trials combining the use of targeted agents along with chemotherapy regimens have shown added benefit of combining such therapeutic

schemes. As a result, many standard protocols for various cancers include the combination of a targeted agent along with conventional chemotherapy. As example, the anti-angiogenic agent, bevacizumab is combined with first-line and second-line chemotherapy (oxaliplatin, 5-fluorouracil, leucovorin (FOLFOX4) in the treatment of metastatic colon cancers. Bevacizumab, along with carboplatin and paclitaxel, is use in the treatment of non-small cell lung cancer (NSCLC). Bevacizumab (Avastin) is a monoclonal antibody that inhibits vascular endothelial growth factor A's (VEGF-A) activation of its target receptors. Though targeted agents have shown great benefit in many cancer types, for EC the use of targeted therapy is absent from standard of care protocols. However, recent advances in the understanding of the signaling pathways that are dysregulated in EC have led to the initiation of several clinical trials that are aimed at evaluating the therapeutic effectiveness of targeted agents in this disease. With the following, we will introduce what is most recently known for targeted agents in Phase II and III clinical trials for recurrent or metastatic EC.

Clinical Trials for Targeted Therapies in Endometrial Cancer

At the molecular level, Type I and Type II ECs are distinct diseases. The most frequently altered signaling pathway in Type I ECs is the PI3K/PTEN/AKT/mTOR pathway, which is dysregulated by oncogenic mutations, PTEN loss of function, and/or overexpression of tyrosine kinase receptors (TKR)s. Type II ECs tend to have inactivation of p53 and/or p16 and genetic instability. Other features frequently observed in Type II ECs are loss of E-cadherin expression and the amplification and overexpression of *Her2/neu*. From these known molecular signatures, three areas of interest, PI3K/PTEN/AKT/mTOR, EGFR and *Her2/neu*, and VEGF, have been the target of many recently completed and ongoing clinical trials for recurrent or metastatic EC.

PI3K/PTEN/AKT/mTOR

Because of the high prevalence of PTEN loss of function (~83%, Type I) and *PI3K* mutations and amplifications (~30%, Type I) in EC, many Phase II and III clinical trials have assessed the effectiveness of agents targeting the PI3K/PTEN/AKT/mTOR. mTOR activation is a key survival pathway for EC cells. Currently completed Phase II clinical trials have assessed mTOR inhibitors, everolimus (391), temsirolimus (392), and ridaforolimus (393), in EC. Complete responses were not found in these studies, however 43% (everolimus), 44% (temsirolimus), and 26% (ridaforolimus) of patients obtained stable disease. These modest responses have encouraged Phase II clinical trials to evaluate the benefit of combining temsirolimus with chemotherapy agents, such as topotecan, paclitaxel, docetaxel, or doxorubicin (reviewed, (390)), in recurrent and metastatic ECs. The combination of PI3K inhibitors along with mTOR inhibitors has recently become of interest. Studies have shown that the loss of negative feedback on PI3K observed with mTOR inhibition can be overcome by dual inhibitors (394). New agents that are dual inhibitors of PI3K-mTOR, XL765 and PKI-587, are currently in Phase I clinical trials (reviewed, (390)).

EGFR and Her2/neu

In EC, EGFR expression is found in 50-80% of cases and is associated with poor clinical outcome (395-397). In Type II ECs, mostly of the non-endometrioid histotype, *Her2/neu* gene amplification (10-30%) (35) and EGFR expression are common (395-397). Type II ECs are the most problematic, as these EC types typically have disease dissemination at initial time of diagnosis and are absent for aberrations in the PI3K/PTEN/AKT/mTOR pathway which is being intensely studied in on-going clinical trials. Antibodies and small molecule inhibitors targeting EGFR and/or Her2/neu and the tyrosine kinase domains of EGFR are established targeted therapies in cancers, such as NSCLC and breast cancer. A Phase II clinical trial examining the

efficacy of the EGFR inhibitor, erlotinib, in recurrent and metastatic ECs was recently completed. Partial response was reported in 12% of patients, and 43% of patients obtained stable disease (398). Interestingly, fluorescence in situ hybridization analysis of tumors from these patients failed to detect *EGFR* amplification, and genomic DNA analysis of the responders showed wild-type *EGFR* (398). Moreover, 28% of patients with stable disease completely lacked EGFR expression (398). An additional recently completed Phase II clinical trial assessing the EGFR monoclonal antibody, cetuximab, as monotherapy in EC patients previously treated with several lines of chemotherapy showed a partial response of 5% and a stable disease response of 10% (399). *Her2/neu* amplification is prevalent in Type II ECs, occurring in 10-30% of cases (35). A Phase II clinical trial evaluating monoclonal *Her2/neu* antibody, trastuzumab as a single agent in patients with *Her2/neu*-amplified ECs failed to show clinical benefit (400). Phase II clinical trials assessing EGFR inhibitor, gefitinib and *Her2/neu* inhibitor, lapatinib are currently enrolling patients. Despite the unremarkable responses seen with EGFR or *Her2/neu* targeted agents, there is consideration that improved responses may occur with new agents that act as dual inhibitors of EGFR and *Her2/neu* (401). Phase II clinical trials assessing dual EGFR and *Her2/neu* inhibitors, lapatinib and BIBW-2992 are currently underway (reviewed, (390)).

VEGF

Angiogenesis is one of the central hallmarks of cancer. Increasing blood supply to a growing tumor not only “feeds” the growing addiction of cancer cells, but additionally provides for their escape from the primary tumor. In EC, increased density of microvessels is associated with poor survival (402). Likewise, the expression of VEGF is strongly correlated with microvessel density and vascular proliferation (403, 404). A Phase II clinical trial evaluating the efficacy of bevacizumab in combination with carboplatin and paclitxel in recurrent or metastatic ECs is underway at this time (reviewed, (390)). Recently or nearly completed Phase II clinical

trials assessing tyrosine kinase inhibitors, sorafenib (405) and sunitinib (406), which additionally target vascular growth factor receptor 2 (VEGFR2), have shown promising results. Partial response with sorafenib was reported in 5% of patients, with 49% of patients obtaining stable disease (405). Preliminary data from sunitinib studies have reported a partial response rate of 15% and stable disease in 20% of patients (406). Both clinical trials consisted of EC patients previously treated with several lines of chemotherapy (405, 406).

Current Areas of Clinical and Translational Interest in the Clinical Management of Endometrial Cancer

Much of the clinical and translational laboratory-based research efforts in EC are aimed at one or several of the following areas: 1.) evaluation of the efficacy of non-contraceptive use of levonorgestrel (LNG)-releasing intrauterine device (IUD) for the use as a treatment alternative for atypical endometrial hyperplasia, a precursor lesion to EC, or low grade ECs; 2.) increased research efforts towards understanding the molecular pathways involved in Type II ECs; 3.) clinical trials designed to determine whether chemotherapy alone, radiation alone, or combination chemotherapy and radiation is optimal for therapy management of recurrent or metastatic ECs and high-risk ECs; 4.) development of biomarker panels that assist with diagnosis and/or serve as indicators of therapy response; and 5.) understanding and developing schemes to overcome chemotherapy resistance (3, 39, 390, 407).

Potential Therapeutic Benefits or Pitfalls of the use of A2BR Agonists in Cancer

Cell-cell adhesion presence is correlated significantly with therapy sensitivity, and more importantly the direct re-establishment of these structures or their individual proteins in resistant cells restores chemotherapy and/or targeted therapy (EGFR inhibitors) sensitivity. Though a highly attractive target because of its potential to resolve a common issue among all

cancers – therapy resistance, targeting cell-cell adhesions and their individual proteins, such as E-cadherin has been a challenge *in vivo*, as no pharmacological agents are currently known to act to re-establish or stabilize these structures or proteins in carcinoma cells. Chemotherapy regimens are heavily relied on in the treatment of metastatic EC, and of great concern, is the high rate of therapy resistance in women receiving these agents. Our interest in the purinergic pathway and its generation of extracellular adenosine was for two primary reasons, 1.) adenosine has a history of inducing barrier function, a homeostatic event related to cell-cell adhesions and 2.) a number of pharmacological agents are currently available and in use in clinical trials for members of this pathway (reviewed, (126)). Therefore, our discovery of adenosine's activity of A2BR to induce filopodia that function to reform (zipper) cell-cell adhesions in carcinoma cells provides the rationale and relevance of pharmacological targeting A2BR in metastatic patients. Most importantly, this provides the a druggable scheme to target cell-cell adhesions in carcinoma cells. The concept of adenosine-mediated cell-cell adhesion in carcinoma cells and its potential for therapy benefit is truly a unique and innovative idea.

The targeting of purinergic pathway members central to extracellular adenosine activity is currently underway in various diseases, such as chronic obstructive pulmonary disease, asthma, Parkinson's and Huntington's disease , and congestive heart failure associated renal failure. In all cases, targeting specific members in these diseases has shown some therapeutic benefit, with pharmacological agents being well tolerated and having low toxicities. Many of these favorable outcomes, however, have come from localized administration approaches, such as those seen with chronic lung diseases – use of an inhaled delivery mechanism. The potential concern with targeting purinergic pathway members in patients with metastatic cancer, specifically targeting A2BR, is whether a systemic approach would be tolerated. Many of these pathway members have ubiquitous expression across a wide variety of different tissues. In addition, systemic administration of drugs targeting the purinergic pathway could exacerbate pre-existing, non-cancer

conditions. For example, adenosine's activation of A2BR in the lungs of asthmatics acts as an irritant and bronchoconstrictor. Many patients with metastatic cancer are older and therefore have cardiovascular and renal disease. Abnormal expression of A2BR, A2AR, and A1R is found among patients with heart and renal disease, and hyper-activity of these receptors is important in the pathophysiologies of these diseases. For these reasons, triggering A2BR or other adenosine receptor subtypes in tissues that are not the intended target can have profound adverse effects, including transient heart block, bronchospasms, and seizures, all of which have been reported in Phase I and II clinical trials for adenosine receptor agonists or antagonists. The pharmaceutical company, Bayer, currently has an oral A2BR agonist. It is unknown, however, if such an agent would be tolerated and beneficial in patients with metastatic cancer. It is likely that local approaches will be needed for restoring adenosine or A2BR activity to carcinoma cells. Preclinical studies are needed to determine these potential concerns, as many previously deemed un-targatable targets, such as ribonucleases, have proved to be not as toxic/systematic detrimental as once thought. Our laboratory is currently moving forward with designing and implementing preclinical studies for this work.

An additional therapeutic potential, is the use of an implantable intrauterine device (IUD) containing an A2BR agonist. This could be beneficial for the subset of women with EC who are non-surgical candidates. An IUD is a small plastic or copper device that is inserted into the uterus and is well known as a method of birth control. Recent modifications to IUDs have been made which include the ability of these devices to deliver pharmaceutical agents to the uterus, especially synthetic progesterone. Delivery of A2BR agonists to uterine confined disease may help inhibit the extra-uterine spread of the disease in these women. Using a mouse model partially deficient for the phosphatase and tensin homolog (PTEN), we are testing the potential benefit of administering adenosine or A2BR agonists directly to the

uterus. Heterozygous PTEN mice develop endometrial hyperplasia at 6-8 months of age (approximately 100% (371)). One fourth of the mice will later develop EC (371). Administration of these purinergic pathway members, either before or preceding hyperplasia development, will allow us to assess whether A2BR agonists can impede disease development or progression.

CHAPTER FOURTEEN

Appendix

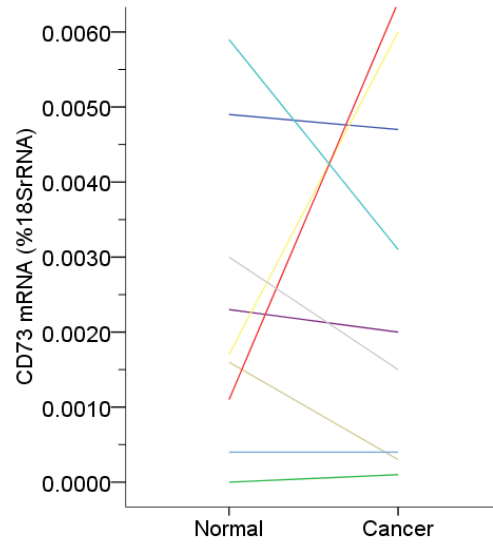
Appendix A

***CD73* is down-regulated or remains unchanged in colon carcinomas**

We have shown that *CD73* down-regulation in carcinomas is not limited to EC. By using qRT-PCR it was shown that the highly aggressive ovarian histotype, HGPSC had significantly reduced levels of *CD73* compared to normal ovary (Specific Aim 1, Figure 5.6). To determine if down-regulation of *CD73* occurred in non-gynecological carcinomas, such as colon, colon carcinomas and patient matched adjacent normal tissue was received from The University of Texas MD Anderson Cancer Center Tissue Biospecimen and Pathology Resource (TBPR) Institutional Tissue Bank and assessed for *CD73* expression. *CD73* transcripts were down-regulated or remained unchanged in the majority of patients.

Figure 14.1. *CD73* expression in normal colon and colon carcinomas. *CD73* transcripts were assessed by qRT-PCR. Transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of *18S* rRNA (%18SrRNA). Individual lines represent a single patient. *CD73* transcripts were down-regulated or remained unchanged in the majority of patients.

Figure 14.1



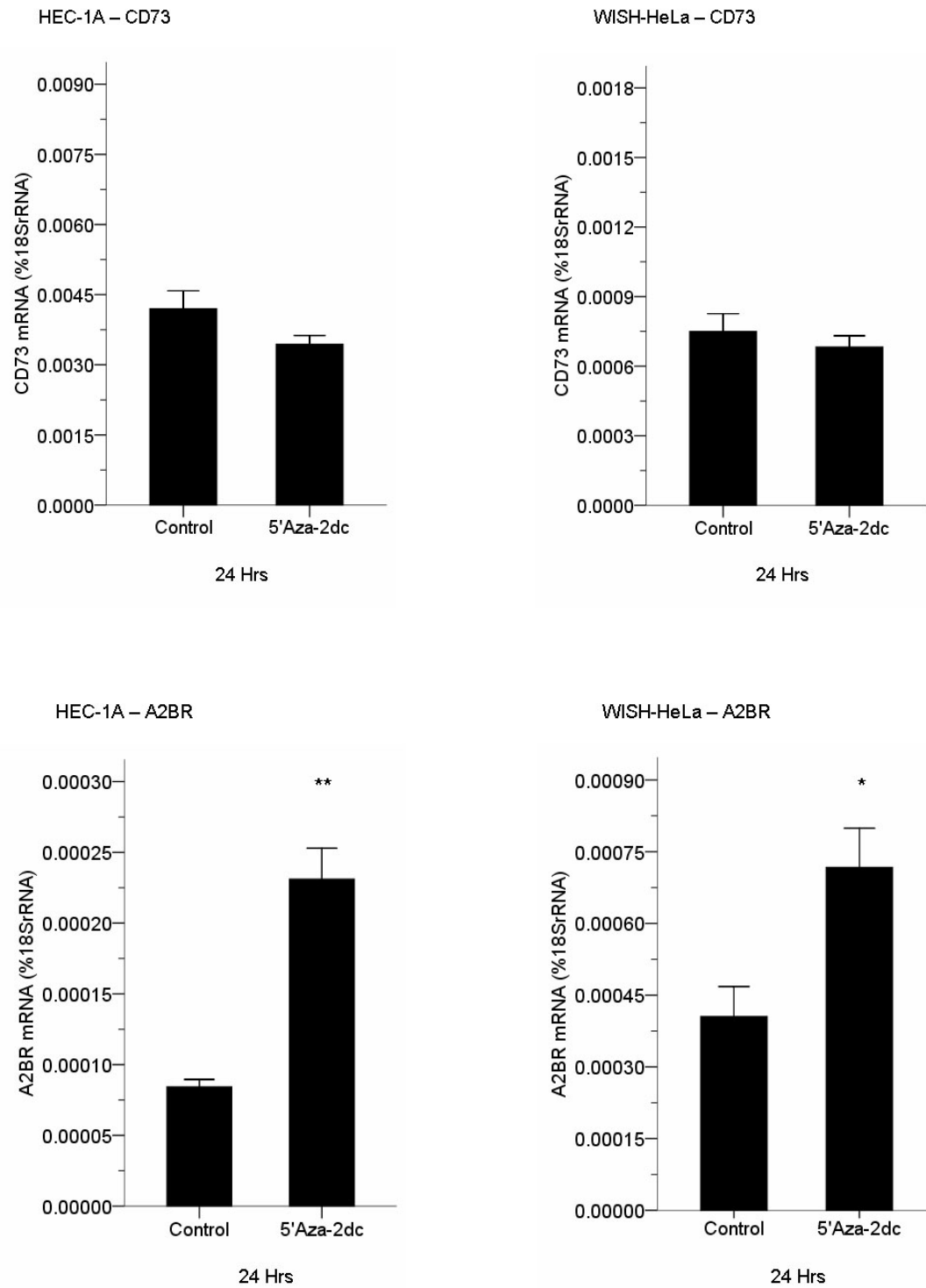
Appendix B

***A2BR* transcripts levels increase with 5-aza-2'-deoxycytidine treatment of hypoxic endometrial carcinoma cells, HEC-1A, and cervical carcinoma cells, WISH-HeLa; *CD73* transcript levels remain unchanged**

Data from specific aim 2, sub-aim 2 (Figure 6.8) showed that hypoxia was ineffective in inducing *CD73* and *A2BR* expression in endometrial carcinoma cells, HEC-1A and HEC-1B. *A2BR* expression increased in HEC-1B cells, but was minimal. DNA methylation of gene promoter regions in carcinoma cells is a common means for silencing unwanted gene expression (reviewed, (408)). We had considered whether methylation of CpG regions near or directly within HIF-1 binding motifs would be a likely event resulting in the inability of hypoxia to induce *CD73* or *A2BR* expression. Using Methylator SVM based software for DNA methylation prediction, courtesy of Harvard Bioinformatics, we identified two predicted methylated cytosines of CpG dinucleotides to fall directly within two HIF-1 binding motifs (5'-A/GCGTG-3') of *A2BR*'s promoter. Treatment of HEC-1A cells and WISH-HeLa cells with DNA demethylating agent 5-Aza-2'-deoxycytidine (5'Aza-2dc) increased *A2BR* transcripts in hypoxia by 3-fold (HEC-1A) and 2.5-fold (WISH-HeLa) (Figure 14.2). *CD73* transcripts remained unchanged. Recall from Figure 6.8, HEC-1A cells, after 24 hours, hypoxia has no effect on *A2BR* expression as compared to normoxia. With 5'Aza-2dc treatment and following 24 hours of hypoxia, a 3-fold induction was seen. WISH-HeLa cells were initially found to have a significant increase in *A2BR* expression at 24 hours (Figure 6.8 – 4-fold). An additional 2.5-fold induction of *A2BR* occurred with 5'Aza-2dc treatment. These data suggest a direct or indirect role of DNA methylation in *A2BR*'s expression in hypoxia.

Figure 14.2. 5-aza-2'-deoxycytidine treatment of hypoxic endometrial carcinoma cells, HEC-1A, and cervical carcinoma cells, WISH-HeLa. HEC-1A and WISH-HeLa cells were treated with demethylation agent, 5-aza-2'-deoxycytidine (5'Aza-2dc), for 24 hours. Cells were subjected to hypoxia conditions (1% O₂, 5% CO₂) during this period. *CD73* and *A2BR* transcripts were assessed by qRT-PCR. Transcript values were normalized to *18S* ribosomal RNA (rRNA) and are presented as molecules of transcript/molecules of *18S* rRNA (%18SrRNA). *CD73* transcripts did not change with 5'Aza-2cd treatment compared to the vehicle control (DMSO), while *A2BR* transcripts increased in HEC-1A and WISH-HeLa. Error bars represent \pm 1 SE. (*p<0.05, **p<0.005)

Figure 14.2



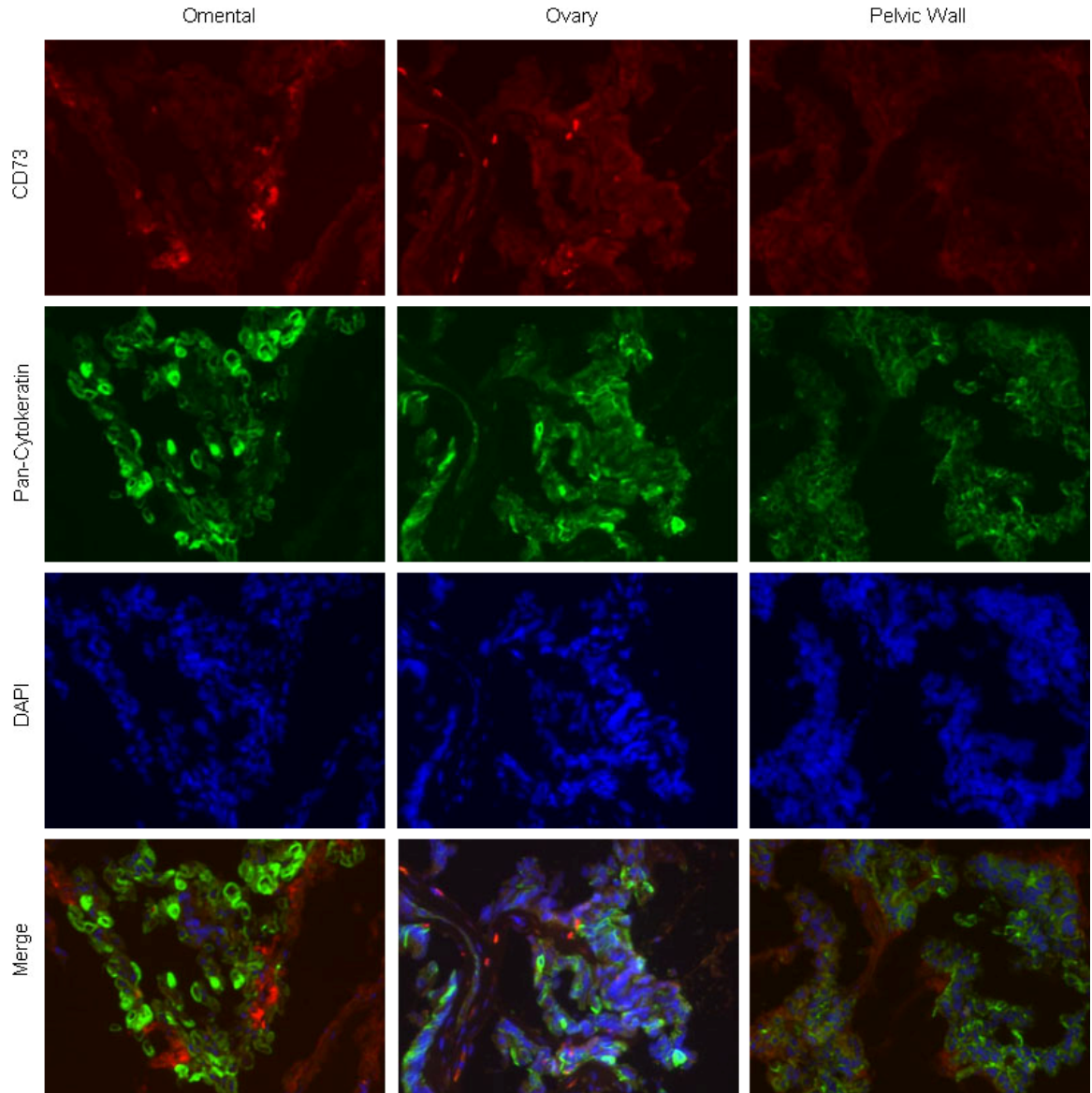
Appendix C

CD73 remains down-regulated in metastatic endometrial carcinoma lesions.

When treating advanced cancer, most often the primary tumor has already been surgically removed. Therefore, treatment is actually directed to metastatic disease. The extent to which a primary tumor resembles metastases at the molecular level is currently controversial. Much of the work that has gone into understanding this issue has come from studies in breast cancer. In a study aimed at determining the reason for trastuzumab failure in metastatic breast cancer patients whose Her2/neu positivity had been determined from the primary tumor, a 15% discordance in Her2/neu expression was found between primary tumors and their corresponding metastasis (409). Because of our interests in the therapeutic potential of CD73's product, adenosine, and its activation of A2BR, we were greatly interested in whether CD73's expression remained down-regulated in metastases of EC. Our previous studies have shown that CD73 is significantly down-regulated in the primary tumors of grade 3 endometrioid endometrial carcinomas (G3 EEC) and uterine papillary serous carcinomas (UPSC). To assess CD73's expression in EC metastases, CD73 expression was assessed by dual labeled immunofluorescence in cryosections of 5 omental metastases, 4 ovary metastases, and 1 pelvic wall metastasis from 10 different patients with a known endometrial cancer primary tumor (Figure 14.3). CD73 expression in the metastasis in all cases was found to be minimal and equal to that of the surrounding tissue. These data suggest that the down-regulation of CD73's seen in EC primary tumors remains down-regulated in its metastases.

Figure 14.3. CD73 and pan-cytokeratin immunofluorescence in metastatic endometrial carcinomas. Cryosections of metastatic endometrial carcinomas were characterized for CD73 expression by immunofluorescence. Pan-cytokeratin served as a marker of carcinoma cells. The low intensity of CD73 expression in these metastases is comparable to that observed in primary tumors (Specific Aim 3, Figure 7.2) Image magnification, 20X

Figure 14.3



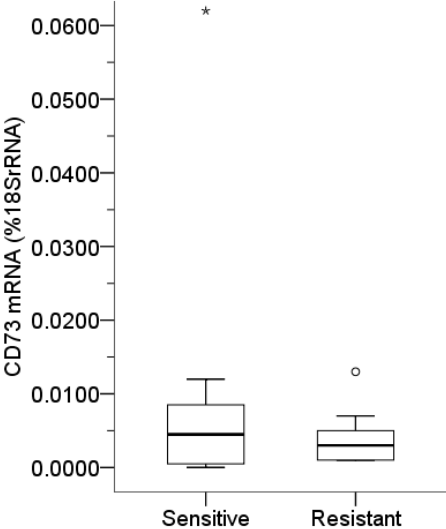
Appendix D

***CD73* expression is no different among platinum sensitive or resistant ovarian high grade papillary serous carcinoma (HGSC) cases.**

Platinum resistance is a major determinant of poor prognosis in patients with ovarian cancer. To determine whether *CD73* is an important molecular determinant of therapy sensitivity, *CD73* transcripts were quantified in platinum sensitive or resistant ovarian HGSC cases. *CD73* expression was not significantly different between the platinum sensitive and resistant groups (Figure 14.4). However, as a group, *CD73* expression in ovarian HGSC is quite low (Specific Aim 1, Figure 5.5), so it may not be reasonable to expect that platinum resistant tumors would have even lower expression of this marker.

Figure 14.4. *CD73* expression in platinum sensitive and resistant ovarian high grade papillary serous carcinoma (HGPC). *CD73* transcript data from qRT-PCR studies of platinum sensitive and resistant high grade papillary serous carcinomas (HGPC) were plotted using a box and whisker plot: bottom box, 25th percentile; top box, 75th percentile; black bar, 50th percentile (median); whiskers, upper and lower adjacent; circles, outside fence. *CD73* transcript values were normalized to 18S ribosomal RNA (rRNA) and are presented as molecules of *CD73*/molecules of 18S rRNA (%18SrRNA). *CD73* transcripts were not significantly different between platinum sensitive and resistant HGPC.

Figure 14.4



CHAPTER FIFTEEN

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CHAPTER SIXTEEN

Vita

Jessica Lynn Bowser was born in Horton, KS, December 6, 1980, to Jerry and Mary Bowser. She graduated from Jackson Heights High School in rural Jackson County, KS. In August, 2001, she signed a national letter of intent with Wichita State University to compete as a student athlete in NCAA Division I track and field. She competed into her first year of graduate school. She received a Bachelor of Science degree in Biology and Chemistry minor in December, 2003, and a Master of Science degree in Molecular Biology with emphasis in Reproductive Endocrinology in May, 2006. She was an adjunct faculty member in the Department of Biological Sciences at Wichita State University until May, 2007. In August, 2007, she entered the Doctoral degree program at the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences where she conducted her studies under the supervision and guidance of Dr. Russell Broaddus, Department of Pathology, the University of Texas MD Anderson Cancer Center. Concurrent with her doctoral training, she received translational research education through the T32 Center for Clinical and Translational Sciences (CCTS) pre-doctoral training program, which is funded by National Institutes of Health Clinical and Translational Award TL1 RR024147 from the National Center for Research Resources. Dr. David Hong, Department of Investigational Cancer Therapeutics, and Dr. Bryan Hennessy, Department of Gynecological Medical Oncology, the University of Texas MD Anderson Cancer Center, served as her clinical mentors for the CCTS. Her work from this dissertation has been presented at, the 101st Annual American Association for Cancer Research, Washington, D.C., April, 2010; the Keystone Symposia, Epithelial Plasticity and Epithelial-to-Mesenchymal Transition, Vancouver, British Columbia, Canada, January, 2011; the 17th Annual Texas Forum for Reproductive Sciences, Houston, TX, April, 2011; and the 102nd Annual American Association for Cancer Research, Orlando, FL, April, 2011.

Graduate Academic Awards:

MD Anderson Alumni and Faculty Association Graduate Student Award in Basic Science Research, First Place, MDACC Trainee Research Day, May, 2011

MD Anderson Alumni and Faculty Association People's Choice Award in Basic Science Research, First Place, MDACC Trainee Research Day, May, 2011

17th Annual Texas Forum for Reproductive Sciences Poster Competition Award Winner, Third Place, March, 2011

MD Anderson Alumni and Faculty Association Trainee Excellence Award, March 2011

The University of Texas Graduate School of Biomedical Sciences at Houston Scientific Conference Travel Award, February, 2011

Keystone Symposia Scholarship, Epithelial Plasticity and Epithelial-to-Mesenchymal Transition, January, 2011

The John P McGovern Presentation Skills Competition Award Winner, First Place, August, 2010

Thomas F Burks Outstanding Leadership and Service Award, May, 2010

Dora Wallace Hodgson Outstanding Master's-Level Student Award, Wichita State University , May, 2006

Annual Graduate Research and Scholarly Projects Poster Competition Award Winner, Runner-up, Wichita State University, April, 2005

NCAA Division I Missouri Valley Conference Academic Scholar, Three Time Award Winner, May, 2004

Athletic Awards during Graduate School:

Wichita State Women's All-Time Outdoor Top 10, Three Time Top 10 Athlete, Women's Heptathlon, Women's Javelin, and Women's Pole Vault, May, 2010

NCAA Division I All Conference Athlete, Missouri Valley Conference, Three Time Award Winner, May 2004

Steve Prefontaine Classic, Oregon University, Runner-up, Women's Pole Vault, April, 2004

NCAA Division I Missouri Valley Women's Conference Championship, Three Time Top Three Award Winner, Women's Heptathlon, Women's Javelin, and Women's Pole Vault, May 2004

NCAA Division I Missouri Valley Conference Women's Conference Championship, Team Champions, May, 2004

NCAA Division I Outstanding Missouri Valley Conference Athlete of the Week, May, 2004

NCAA Division I Regional Qualifier, Two Time Qualifier, Women's Javelin and Women's Pole Vault, May, 2004