WNT/NFKB CROSSTALK MAY DOWNREGULATE CYTOPLASMIC B-CATENIN LEADING TO INDUCTION OF EMT.

By

Geoffrey Alexander Wilt

BA Biochemistry (Hood College) 2007

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

For the degree of

MASTER OF SCIENCE

In

BIOMEDICAL SCIENCE

In the

GRADUATE SCHOOL

Of

HOOD COLLEGE

May 2018

Accepted:

_____	_____
Raymond K. Blanchard, Ph.D. Committee Member	Ann L. Boyd, Ph.D. Director, Biomedical Science Program
_____	
James M. Cherry, Ph.D. Committee Member	
_____	_____
Ricky Hirschhorn, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

STATEMENT OF USE AND COPYRIGHT WAIVER

I do authorize Hood College to lend this mock grant proposal, or reproductions of it, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

DEDICATION

This is dedicated to my grandmother.

ACKNOWLEDGEMENTS

I would like to thank the many people who aided in proofreading and editing, not least among them Dr. Raymond Blanchard and Dr. Ricky Hirschhorn. I would also like to thank my parents for their constant support and encouragement.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

LEAVE BLANK—FOR OFFICIAL USE ONLY.

Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
WNT/NFKB CROSSTALK MAY DOWNREGULATE CYTOPLASMIC B-CATENIN LEADING TO INDUCTION OF EMT.

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Wilt, Geoffrey Alexander	BA Biochemistry	N/A

3c. POSITION TITLE
Graduate Student

3d. MAILING ADDRESS *(Street, city, state, zip code)*
401 Rosemont Ave.
Frederick, MD 21201

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: N/A FAX: N/A

E-MAIL ADDRESS:
Gaw3@hood.edu

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☒ No ☐ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
06/01/2018	06/01/2021	$100,000	$150,000	$300,000	$450,000

9. APPLICANT ORGANIZATION
Name: Geoffrey Alexander Wilt
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Geoffrey Alexander Wilt
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail: Gaw3@hood.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Geoffrey Alexander Wilt
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail: Gaw3@hood.edu

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13.
(In ink. "Per" signature not acceptable.)
N/A

DATE

PROJECT SUMMARY (See instructions): 200 word max

Molecular interactions in the cell are not bound by discrete cellular pathways; there is significant communication between the pathways leading to tightly regulated processes. The current research will examine if Wnt and NFκB pathway component interplay can stimulate endothelial to mesenchymal transition (EMT) via the cell-junction component β-catenin (*Ctnn-B*). The classical Wnt pathway utilizes *Ctnn-B* as a signaling molecule; specifically, under activated canonical Wnt signaling to induce development. Cell-cell structure is maintained through various junctions, such as adherens junctions which utilize *Ctnn-B* to connect the cytoskeleton to E-cadherin, a transmembrane protein. Recently, a novel mechanism has been proposed wherein the NFκB pathway can also be inhibited by *Ctnn-B*. To determine adverse effects of Wnt/NFκB crosstalk on EMT, a previously developed Wnt signal sensor cell line was used to demonstrate five genes with changes in expression common to both Wnt and NFκB pathways. These genes will be silenced utilizing siRNA, after which a Wnt signal will be induced and western blotting will demonstrate any adverse alterations of Wnt/NFκB signaling capable of altering expression of *Ctnn-B*. Subsequent cell motility assays will demonstrate that Wnt/NFκB pathway alteration observed from western blotting can promote EMT to prove that pathway changes ultimately impact cellular physiology.

RELEVANCE (See instructions): 60 word max

Cell division and EMT are essential to proper tissue development. Disruption of these processes contributes to tumor growth. The immune system is capable of limiting abnormal growth, however immunosuppression can lead to tumor formation. A further understanding of the interplay between the Wnt and NFκB signaling pathways, and the EMT process could reveal targets for earlier diagnosis of metastasis, leading to earlier intervention.

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name: QIAGEN Sciences			
DUNS:			
Street 1: 6951 Executive Way		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: USA		Zip/Postal Code: 21703
Project/Performance Site Congressional Districts: 6th Congressional District			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Geoffrey Alexander Wilt	Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hood College, Frederick, MD	B.A.	2007	Biochemistry
Hood College, Frederick, MD	M.S.	2018 (anticipated)	Biomedical Science: concentration Molecular Biology

A. Positions and Honors

Associate Scientist II, QIAGEN Sciences

B. Selected peer-reviewed publications (in chronological order)

C. Research Support

D. Selected non-peer-reviewed publications (in chronological order)

Geoffrey Wilt, Qiong Jiang, Vikram Devgan. *Cross-regulation of Wnt signaling by NFκB pathway components.* Program# 881, Poster B1338. ASCB, 2010.

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College

QIAGEN Sciences

Clinical:

Animal:

Computer:

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges for pelleting cell lysates
- Applied Biosystems 7900 PCR machine for RT-qPCR assays
- EnVision 2103 Multilabel Reader for Luminescence measurement
- CO_2 Incubators for cell culture maintenance
- Fluorescent Microscope for cell observation
- XCell SureLock™ Mini-cell and XCell II™ Blot module for western blotting gel and transfer
- Plate shakers for western blotting membrane washing
- Tecan plate reader for Fluorescence measurement

The following consumables will be purchased with grant funding:

- Antibodies (AbCam)
- Dulbecco's Modified Eagle's Media (ATCC®)
- FluoroBlok™ inserts (Corning)
- Defined Fetal Bovine Serum (VWR/HyClone™)
- Dual-Luciferase® Reporter Assay System (Promega)
- siRNA assays (QIAGEN)
- HiPerfect Transfection reagent (QIAGEN)
- RNeasy Plus 96 RNA isolation kit (QIAGEN)
- RT^2 SYBR® Green qPCR mastermix (QIAGEN)
- RT^2 Profiler gene expression assays (QIAGEN)
- Protein ladders (ThermoFisher)
- NuPage™ 8% Bis-Tris gel (ThermoFisher/Invitrogen™)
- MEM Non-Essential Amino Acids (ThermoFisher/Gibco™)
- Cell culture dishes and plates (VWR)

SPECIFIC AIMS

Dismantling of adherens junctions releases β-catenin (*Ctnn-B)* from cytoskeletal structures into the cytoplasm where it participates in various cellular pathways. In classical Wingless-type MMTV integration site (*Wnt*) signaling, *Ctnn-B* plays a crucial role for regulation of gene expression relating to development. Research has suggested that *Ctnn-B* also plays a role in suppression of NFκB signaling related to immune-response. Careful regulation of *Ctnn-B* is needed to coordinate the Wnt and NFκB pathways in conjunction with Endothelial to Mesenchymal transition (EMT). As cell signaling is not a closed system and relies on crosstalk of multiple pathways, we hypothesize that Wnt/NFκB crosstalk can regulate cytoplasmic levels of *Ctnn-B* leading to stimulation of EMT.

Aim 1) **To demonstrate that siRNA knockdown of any of the five gene products functioning in both Wnt and NFκB pathways deregulates *Ctnn-B* localization, yielding an increase in nuclear *Ctnn-B*.**

A) Levels of mRNA will be measured after treatment of cells with pools of siRNA designed for single gene knockdown using reverse-transcription quantitative polymerase chain reaction assays.

B) Western blotting will assess levels of *Ctnn-B* as well as target genes in four distinct intracellular localization protein fractions after centrifugal cell fractionation of cell lysates.

Aim 2) **To demonstrate that lower cytoplasmic levels of *Ctnn-B* results in stimulation of EMT leading to an increase of cell motility.**

A) Cell invasion assays will be utilized to demonstrate that gene expression alteration is associated with an increase in cell motility.

BACKGROUND AND SIGNIFICANCE

Introduction

There were nearly 230,000 new cases of breast cancer diagnosed in 2015 with approximately 40,000 deaths in the United States alone (SEER 2015). Looking at the 5-year survival rates, patients with metastatic diagnosis have a poor prognosis, with only 25% survivability. With these statistics in mind, diagnosing patients earlier, and with increased precision, could lead to improved intervention times. Regrettably, most current methods used for diagnosis only detect metastasis after it has already occurred. As with all aspects of cancer, metastasis results from perturbation of normal cellular pathways. Included among these pathway perturbations is an increase in cell growth due in part to altered Wnt pathway signaling. Research also suggests that immune suppression could allow tumors to grow and evade apoptosis (Kaler *et.al.* 2009; Khanbolooki *et.al* 2006; Krelin *et.al.* 2007; Roy *et.al.* 2017; Pai *et.al.* 2017). Following evasion of apoptosis, activation of the endothelial to mesenchymal transition (EMT) leads to the tumor cells' ability to detach from primary sites, migrate through the body, and potentially seed new tumors.

Interactions within the cell are a careful balancing act, which when altered can lead to varied disease states. This is highly evident in the case of cancer, where a multitude of changes in the cell causes unchecked growth. Hanahan and Weinberg proposed multiple universal characteristics of tumor growth, one of which is the ability of a tumor to invade surrounding tissue (2000). Invasion is assisted through a change away from an endothelial phenotype, which is closely related to tissue structure, to a more motile phenotype akin to mesenchymal cells (Howard *et.al.* 2011; Terry *et.al.* 2017).

These cells are capable of detaching from tissue and can differentiate into any number of specialized cells. Differentiation into specialized cells is a normal developmental process, however, it is usually tissue specific. Metastasis has been shown to involve EMT, the process through which cells detach from the primary site and become free-floating in the bloodstream and lymph system. These free-floating cells have the potential to develop into additional tumors by seeding in a different tissue.

A second aspect proposed by Hanahan involves a tumor's ability to evade apoptosis through immune system suppression (2000). In recent years, the relationship between the immune system and tumor growth has been increasingly examined, leading to the growing field of immuno-oncology. This area of research focuses on the ability of the immune system to recognize and target various aspects of tumor growth (Finn 2012, Ma and Hottiger 2016). One of the key pathways in immunology is the NFκB pathway. Various interactions at the cell surface are responsible for the cells ability to recognize aberrant cells and then induce apoptosis. These aberrant cells include senescent or dead cells, and cells with abnormal growth properties, commonly referred to as neoplastic growth. In tumors, however, the immune response can be blunted allowing for neoplasia, which has the potential to develop into tumors. Another characteristic of cancer proposed by Hanahan pertains to a tumor's ability to acquire and sustain insensitivity to anti-growth signals (2000). These anti-growth signals involve either differentiation into specialized tissues, or entrance to a quiescent state, both of which curtail excess cell growth (Cadigan and Waterman 2012). Intercellular signaling relies on extracellular signaling molecules including small proteins such as cytokines. The gene products of the *Wnt* family of genes are among the most highly characterized cytokines (MacDonald and

He 2012; Willert and Nusse2012). The Wnt signaling pathway typically enhances cell growth or alteration of the cell-cell microenvironment to enhance tissue generation. In the off-state of the Wnt pathway, there is no external signal, leading to lower cytoplasmic levels of β-catenin (*Ctnn-B*) and no enhanced cell growth signaling. When a Wnt cytokine binds to surface receptors, however, a positive signal is received by the cell and a kinase cascade is initiated. This cascade ultimately results in an increase in cytoplasmic *Ctnn-B* leading to its translocation into the nucleus where *Ctnn-B* interacts with various transcription factors and increases gene expression for cell growth related proteins (Rao and Kühl 2010; Valkenberg *et.al.* 2011). In tumors, however, regulators of Wnt activity are bypassed allowing for a continuous activated signal leading to unchecked cell growth (Pećina-Šlaus 2010).

Mechanisms of the Wnt pathway

Properly regulated Wnt signaling allows for wound healing, embryogenesis, and organ development, while perturbation of the Wnt pathway plays a key role in tumorigenesis (Rao and Kühl 2010). The Wnt pathway has three distinct signaling methods; the canonical β-catenin and non-canonical Ca^{2+} signaling pathways, and the planar cell polarity cytoskeletal regulation (Valkenburg 2011). Included in the canonical Wnt pathway are many structural proteins, cytokines, and activated genes; from Wnt signaling molecules, to transmembrane proteins of the Frizzled (*Fzd*) family proteins, as well as many cytoplasmic proteins, including *Ctnn-B*, glycogen synthase kinase-3β (*GSK3B*), axis inhibitor (*Axin*), and adenomatous polyposis coli (*APC*) (Rao and Kühl 2010; Willert and Nusse 2012; Cadigan and Waterman 2012).

Current research has found that *Ctnn-B* plays a key role in the Wnt signaling pathway. Signaling begins via the *Wnt* family of genes; in the absence of this signal, *Ctnn-B* is phosphorylated by an *Axin/APC* containing complex. *GSK3B* is also associated with this complex, which will be targeted by a Wnt signal (Espinosa *et.al.* 2003). When there is an increase in *Ctnn-B* phosphorylation, interactions with β-transducin repeat containing protein (*β-TRCP*) increase ubiquitination of *Ctnn-B* leading to its degradation (Espinosa *et.al.* 2003). In the non-activated Wnt state, most *Ctnn-B* is associated with cell-cell junctions, such as adherens junctions.

In canonical Wnt signaling, a *Wnt* ligand will bind to a *Fzd* receptor leading to activation of Dishevelled (*Dsh*) genes. Activation of these genes will increase phosphorylation of *GSK3B*, leading to its inability to aid in phosphorylation of *Ctnn-B* (Rao 2010, Espinosa *et.al.* 2003). Due to this decrease in phosphorylation, *Ctnn-B* will not be targeted for degradation, leading to an increase in cytoplasmic levels. This will lead to an increase in translocation of *Ctnn-B* from the cytoplasm into the nucleus where it complexes with the DNA binding proteins T-cell factor (*TCF*) and lymphoid enhancing factor (*LEF*) (Cadigan andWaterman 2012). These transcription factors are typically associated with sequence specific promoter regions, which can influence gene expression either through an increase when the *TCF/LEF-Ctnn-B* complex is active or a decrease when the regions are not accessible. Among the genes affected by this promoter element are those involved in extracellular matrix (ECM) and cell growth, including fibronectin (*FNI*), *c-myc*, *c-jun*, and peroxisome proliferator-activated receptor-delta (*PPAR-δ*) (Weeraratna *et.al.* 2002). These genes are related to cell-growth signaling and with an increase in their activity a cell will go from a quiescent state to a growth state.

Mechanisms of the Endothelial to Mesenchymal Transition

Proper intercellular connections are critical for correct cell growth and development; these junctions contain transmembrane proteins which span the cell membrane and can connect to peripheral membrane proteins in the cytoplasm, thereby allowing cell-cell communication as well as interactions between the extra-cellular matrix and the cytoplasm of the cell (Kissin *et.al.* 2002). This allows cells to form an epithelial barrier between the extra-cellular matrix and the inner-tissue basement. This gradient is known as apical-basal polarity, and is a characteristic of endothelial cells (Lamouille *et.al.* 2014). Cells with this phenotype are in the lining of the intestines, or the epithelial cells of the skin; conversely, mesenchymal cells lack organized cell junctions as well as apical-basal polarity, resulting in their increased motility.

Occasionally cell-cell connections can be lost through normal pathways; however, abnormal loss of these junctions is crucial for tissue invasion and metastasis. This phenotypic change, known as endothelial to mesenchymal transition and abbreviated EMT (Gordon *et.al.* 2008), consists of several different cellular functions. For example, Type I EMT is typically involved in proper development of embryos while Type II aids in proper wound healing. Type III EMT however has been determined to be closely associated with metastasis (Lamouille *et.al.* 2014).

In each of these transitions, cell growth is activated and new cells move to occupy different spaces in the tissue. For Type I and Type II, this is a normal process allowing for proper development to occur or for wounds to heal properly. With Type III transition however, cell growth continues unabated leading to tumor growth and tissue invasion. A hallmark of Type III EMT is that cells gain the ability to detach from the basement

membrane of the originating tissue, traversing the bloodstream and lymph system, and finally seeding itself onto a new site.

In order to transition from an epithelial type into a mesenchymal type several changes must occur. Among these, gene expression must favor mesenchymal phenotype, cytoskeletal structural changes must occur, and cell-cell junctions must be lost. This is accomplished, in part, through a loss of adherens junctions cell-cell adhesion complexes (Sulzer *et.al.* 1998). Typically, these junctions form the basis of connections to the actin cytoskeleton and are comprised of the surface marker E-cadherin and *Ctnn-B,* which acts as a bridge with various other cytoplasmic proteins to connect to the cytoskeleton. During EMT, these junctions are broken down with *Ctnn-B* being targeted for degradation while E-cadherin can be returned to the cell surface or targeted for degradation (Palacios *et.al.* 2005). Not only does this allow a cell the capacity to recycle structural components to different areas of the cell, but it allows for tissues to replace apoptotic and senescent cells.

Mechanisms of the NFκB pathway

One well-established component of the immune system is the NFκB signaling pathway. Originally discovered close to 30 years ago, the *NFκB* family of transcription factors is involved in several cellular functions including inflammation and programmed cell death, known as apoptosis (Dinarello 1996; Sun *et.al.* 2005; Hayden and Ghosh 2011). This family of transcription factors exhibits differential binding specificity based on the subunit combinations of hetero- and homodimers of *NFκB1, NFκB2*, *RelA*, *RelB,* and *c-rel* (Liou 2002). The immune system utilizes a signal cascade involving many surface receptors and cytokines to aid in distinguishing between self and non-self

material that must be targeted for degradation. One of several signaling cascades involved with the NFκB pathway is the cytokine interleukin-1β (*IL-1β*) and its associated receptor Interleukin-1 receptor, type 1 (*IL-1R1*). The *IL-1* family of cytokines is not normally expressed in healthy cells; rather it is expressed as a response to inflammatory stimuli, including tumor cells (Dinarello 1996; Spriggs *et.al.* 1990; Rajasekhar *et.al.* 2011).

Activation of an NFκB signal is accomplished via various cascades initiated by extracellular signals; the two most potent signals being Tumor Necrosis Factor (*TNF*) and *IL-1* (Spriggs *et.al.* 1990; Pomerantz and Baltimore 2002). In order to prevent unregulated transcriptional activity, the *NFκB* family dimers are sequestered in the cytoplasm via a family of seven inhibitors, *IκBα*, *IκBβ*, *IκBγ*, *IκBε*, *BCL3*, *p100*, and *p105*. These inhibitors bind via non-covalent interactions and as such can be affected by phosphorylation (Sun 2012). When a cytokine binds to its associated receptor, such as *TNF* binding to Tumor Necrosis Factor receptor super-family member 10A (*TNFRSF10A*), otherwise known as Death Receptor 4 (*DR4*), or such as IL-1 to Interleukin-1 receptor (*IL-1R1*), signal transduction kinases are activated leading to phosphorylation of *IκB* members (Liou 2002).

Upon phosphorylation, the inhibitors dissociate from the *NFκB* dimer allowing its translocation into the nucleus where the dimer acts on specific sequences to increase transcription. This increases expression of apoptosis and immune response genes. Interestingly, one currently known transcriptional target also includes Cyclin-D1 (*CCND1)*, which is a key regulator of the G1 cell cycle control point, resulting in an

increase of cell growth (Hinz *et.al.* 2007) and showing that signal cascades affect multiple signal pathways.

Pathway crosstalk

In the tumor microenvironment, it was determined that *IL-1β* was required for inactivation of *GSK3β*. While *GSK3β* is active, *Ctnn-B* in the cytoplasm is phosphorylated, which leads to destabilization and is then targeted for ubiquitination and degradation (Rao 2010). Phosphorylation of *GSK3β* via *AKT* however leads to dissociation of *GSK3βB* from *Ctnn-B* complex leading to a different conformation of *Ctnn-B* that is typically translocated to the nucleus where it interacts with various transcription factors. Among these transcription factors are the T-cell factor/lymphoid enhancer-binding factor (*TCF/LEF*) family, which increase gene expression of *Wnt* and other cell cycle related genes (Katoh and Katoh 2009). This creates a feedback loop in the Wnt pathway that promotes tumor growth.

Recently discovered, though not completely understood, is the ability of various cancers to not only suppress the immune system, but also to make it operate in their favor (Finn 2012, Linde *et.al.* 2018). To accomplish this, the tumor microenvironment is typically altered to favor growth and evade the macrophages of the immune system. Macrophages, a subset of white blood cells, present various antigens on their cell surface which interact with the NFκB pathway as part of the immune response. Research by Pawan Kaler has shown that NFκB activity, specifically activation of *PDK1* and *AKT*, in macrophages was required to inhibit *GSK3β* thereby increasing Wnt activity (2009). Pro-survival has also been shown possible through *RelB/p50* components of the NFκB pathway (Roy *et.al.* 2017)

Cellular pathways are not strictly closed circuits however, and a significant amount of crosstalk occurs in the cell between all of its many components. The NFκB signaling pathway has significant communication with the Wnt signaling pathway (Kaler *et.al.* 2009; Naskar *et.al.* 2014). As with NFκB signaling pathway, the Wnt signaling pathway is also involved with many cellular functions, chief among them as proper differentiation and development. One of the key components in canonical Wnt signaling is the ligand *Ctnn-B*. This ligand plays a dual role in canonical Wnt signaling; when stable, it is transported to the nucleus to stimulate the *TCF4/LEF* transcription factors for gene expression, however in the cytoplasm it is typically degraded or involved with intercellular junctions (Howard *et.al.* 2011). With unaltered Wnt signaling, cells undergo development however, when this pathway is altered, growth proceeds unhindered, which is a contributing factor in cancer.

E-cadherin links *DR4* to the cytoskeleton allowing for suppression of apoptosis through high expression of E-cadherin (Lu *et.al.* 2014). Epithelial cancer cells have greater sensitivity to *DR4/DR5* mediated apoptosis. As E-cadherin is lost, sensitivity is decreased leading to greater suppression of apoptosis. EMT is partially mediated through a loss of E-cadherin and recycling of Ctnn-B (Orsulic *et.al.* 1999; Kam and Quaranta 2009; Zheng *et.al.* 2011).

Beta-catenin has been shown to bind with *NFκB* dimers (Deng *et.al.* 2002). This leads to a reduction in DNA binding ability of *NFκB*, and lower gene expression of *NFκB* target genes, yielding a blunted immune response and suppression of apoptosis.

Rationale

Gene expression is controlled through several checkpoints; DNA transcription, mRNA translation, post-translational protein modification. The cell environment is a highly complex system with many signaling cascades functioning in concert to control cell growth and development. *Ctnn-B* has been shown to have an active role in several pathways and so we propose to determine whether the cross-talk between multiple pathways can increase EMT activity through modulation of *Ctnn-B.* This would facilitate immuno-oncology through the identification of surface receptors or internal components to target for early metastatic detection.

The immune system receptors are anchored on the cell surface and interact directly with the extracellular matrix. Cytoskeletal structure links the cytoplasm to the cell surface and also forms cell-cell junctions required for tissue integrity. *Ctnn-B* is a known component of the cytoskeletal structure, plays a key role in development through gene regulation, and has also been shown to potentially regulate NFκB signaling; therefore, it must be highly regulated. Wnt and NFκB pathways are known to regulate each other; it is possible that this regulation can also impact cytoskeletal regulation through *Ctnn-B* thereby potentially altering EMT.

PRELIMINARY REPORT / PROGRESS REPORT

Previously, we demonstrated that sensor signal cell lines with luminescent output can be produced to exhibit cross-talk between cellular pathways. Our preliminary data shows that by using siRNA targeted for NFκB components, Table 1, in a Wnt signal sensor line in HEK293 cells, followed by a lithium chloride treatment to stimulate a Wnt signal, luminescence is altered. Negative regulation is indicated by green bars displaying at least 2x fold increase in luminescence, positive regulation is indicated by red bars displaying at least 0.14x decrease in luminescence, and no change as indicated by blue bars. Genes found to modulate this luminescence are the adaptor protein Fas-associated death domain (*FADD*), the surface receptor Tumor Necrosis Factor Receptor super-family member 10A (*TNFRSF10A/DR4*), and RAC-alpha serine/threonine-protein kinase (*Akt1*), which appear to be negative-regulators of Wnt activity as well as *RelA* and *TLR1*, possible positive-regulators of Wnt activity, as shown in Figure 1 (Wilt *et.al.* 2010).

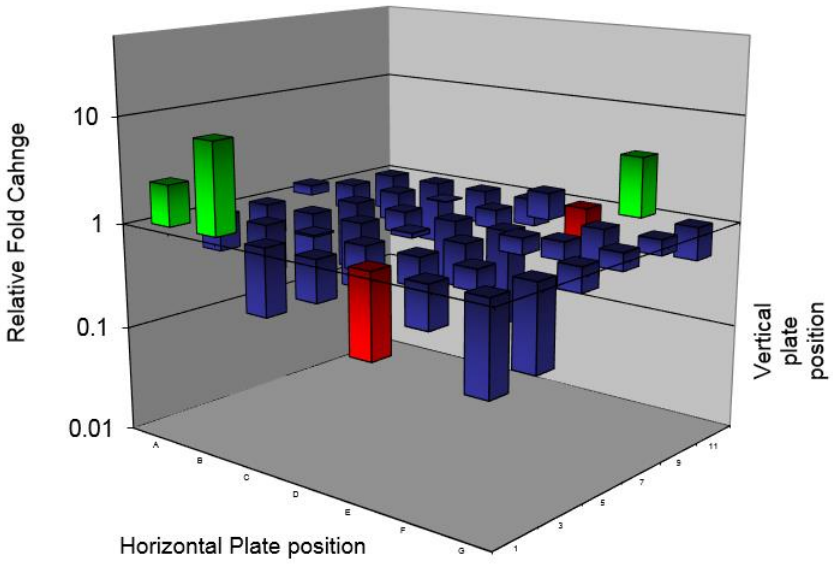

Figure 1: SureSilencing™ Human NFκB Signaling Pathway siRNA Array was used to transfect the Wnt signaling pathway sensor cell line. At 48 hours after transfection, cells were treated with 20 mM LiCl for 6 hours. Dual Luciferase assay was performed and pathway reporter activity values are expressed as relative fold change against cells transfected with negative control siRNA (control).

Table 1. Array Layout: Human NFκB Signaling Pathway siRNA array

	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1	AKT1	BCL10	BCL10	NFκB1	NFκB1	CD40	CD40	CHUK	CHUK	ELK1	ELK1
B	FADD	FADD	IKBKB	IKBKB	IKBKE	IKBKE	IKBKG	IKBKG	IL1R1	IL1R1	IRAK1	IRAK1
C	IRAK2	IRAK2	LTBR	LTBR	MALT1	MALT1	MAP3K1	MAP3K1	NFκBIB	NFκBIB	MAP3K3	MAP3K3
D	MAP3K7	MAP3K7	MYD88	MYD88	NFκB1A	NFκB1A	PIK3CG	PIK3CG	PRKCQ	PRKCQ	EGF4	EGF4
E	RELA	RELA	RELB	RELB	RIPK1	RIPK1	TICAM	TICAM	TICAM2	TICAM2	TLR1	TLR1
F	TLR2	TLR2	TLR3	TLR3	TLR4	TLR4	IGF1R	IGF1R	NFκB2	NFκB2	TNFRSF10A	TNFRSF10A
G	TNFRSF10B	TNFRSF10B	TNFRSF1A	TNFRSF1A	TRADD	TRADD	TRAF2	TRAF2	TRAF3	TRAF3	TRAF6	TRAF6
H	NEG	NEG	NEG	NEG	NOR	NOR	NOR	NOR	TEC	TEC	NOC	NOC

The SureSilencing™ Human NFκB Signaling Pathway siRNA Array was used to analyze the regulation of intracellular Wnt signaling activity by NFκB pathway components. The siRNA array contains duplicate wells of siRNA pairs for 42 different genes specific to the NFκB pathway plus controls.

RESEARCH DESIGN / METHODS

Metastasis results from deregulation of several pathways including Wnt and EMT. The immune system is capable of recognizing these aberrant cells, however can be deregulated thereby resulting in a tumor's ability to evade apoptosis, the first stage of metastasis. There is significant crosstalk between pathways and so the current study aims to demonstrate Wnt/NFκB pathway crosstalk is capable of altering β-catenin levels sufficiently to up-regulate EMT.

Aim 1) **To demonstrate that siRNA knockdown of any of the five gene products functioning in both Wnt and NFκB pathways deregulates β-catenin localization, yielding an increase in nuclear β-catenin.**

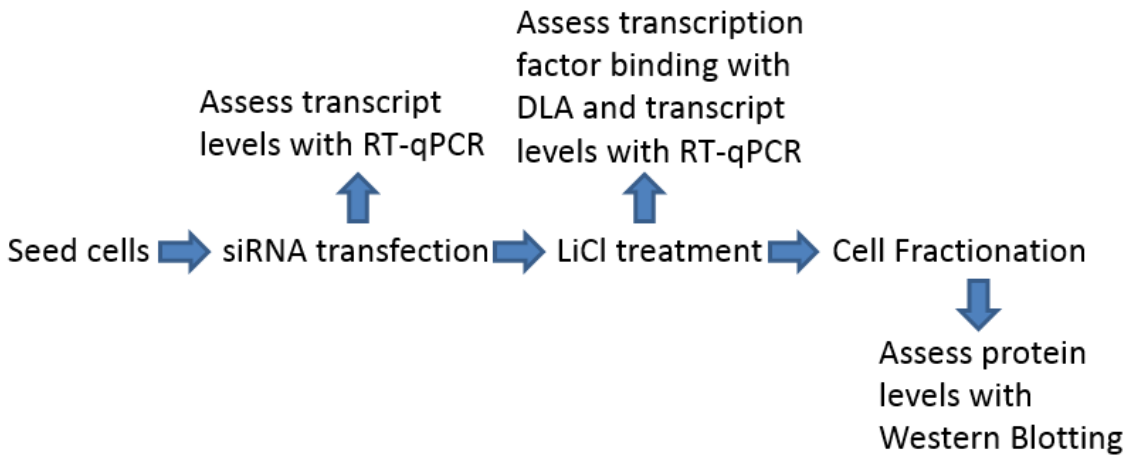

Figure 2: Overview of design for Aim 1.

Previously, we have demonstrated that gene products in Table 2 contribute to crosstalk between the Wnt and NFκB signaling pathways (Wilt *et.al.* 2010). Using siRNA, gene expression of these genes will be knocked down resulting in alteration of Wnt and NFκB pathway crosstalk. Sensor signal cells will be subjected to a pool of siRNA for each gene, commercially-available through QIAGEN, to ensure complete knockdown of target genes. Each pool is composed of four preselected siRNAs for a

target gene in order to ensure complete knockdown. Previously generated signal sensor cells (Wilt *et.al.* 2010) will then be treated with lithium chloride (LiCl) to stimulate a Wnt signal (Klein) and a dual luciferase assay will be performed to confirm successful knockdown. Verification of siRNA knockdown and the RNA level will then be

Table 2: Target genes for siRNA knockdown

UnigeneID	RefSeqID	Gene	Description
Hs.525622	NM_005163	*AKT1*	V-akt murine thymoma viral oncogene homolog 1
Hs.86131	NM_003824	*FADD*	Fas (TNFRSF6)-associated via death domain
Hs.502875	NM_021975	*RELA*	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
Hs.654532	NM_003263	*TLR1*	Toll-like Receptor 1
Hs.591834	NM_003844	*TNFRSF10A*	Tumor necrosis factor receptor super-family, member 10a

Previously determined gene crosstalk between WNT and NFκB pathways to be utilized in gene knockdown to determine alteration of localization of β-catenin.

performed via commercially available reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays from QIAGEN which operate based on the double-strand DNA (dsDNA) binding dye SYBR® Green. Immuno-blotting after isolation of intracellular protein fractions of siRNA treated and LiCl treated Wnt signal sensor cells will demonstrate that altered Wnt/NFκB crosstalk leads to increased degradation and altered localization of β-catenin.

Wnt signal sensor cells will be maintained in DMEM media supplemented with 10% Fetal Bovine Serum (FBS) and 1X Non-Essential Amino Acids (NEAA) in a 5% CO_2 environment at 37˚C. By transfecting these cells with siRNA, knockdown of gene expression will occur as mRNA levels of specific genes are decreased, ultimately yielding a change in pathway activity.

Conditions of optimal siRNA knockdown will be determined including concentration of siRNA as well as time of siRNA treatment. QIAGEN recommended guidelines indicate that $2x10^4$ cells should be seeded per well in a 96-well plate and

subjected to 48-hour transfection of siRNA at 25nM concentration. These concentrations will be included in optimization.

Optimization study for siRNA conditions will be tested in nine separate 96-well plates and each condition run in triplicate. Each plate will include 5nM, 10nM, 25nM, and 50nM siRNA. Plate 1-3 will be treated for 24 hours, plate 4-6 treated for 48 hours, and plate 7-9 treated for 72 hours. Controls will include no siRNA mock transfection as negative control, and QIAGEN AllStars Hs Cell death control siRNA as positive control for transfection efficiency. Final sample count is shown in Table 3.

Table 3: Samples for siRNA optimization per plate

Sample name	Sample type
AllStars Hs Cell death	Positive control
Mock transfection	Negative control
Target siRNA 5nM	Test sample
Target siRNA 10nM	Test sample
Target siRNA 25nM	Test sample
Target siRNA 50nM	Test sample

Optimization using 24-hour, 48-hour, and 72-hour treatment of siRNA from Table 2. Each sample run in triplicate.

Successful transfection with AllStars control should yield increased cell death, visible under light microscopy while the mock transfection should have no effect. We will demonstrate siRNA effectiveness through use of RT-qPCR assays for each target gene. Based on previous experience, we expect a linear increase in Ct with higher siRNA concentration correlating to a decrease in gene expression.

Through stimulation of Wnt signal sensor cells with LiCl (Abu-baker *et.al.* 2013), we can induce Wnt pathway activity. Stimulation with LiCl will utilize previously

determined treatment concentration of 20 mM (Wilt *et.al.* 2010) against triplicate plates different times of treatment; 6-hour, 12-hour, 24-hour, and 36-hour. This will determine the ideal treatment time to induce a Wnt signal. Wnt signal sensor cells will be plated in 96-well culture dishes at $2.0x10^4$ cells per well and allowed to grow overnight before treatment. After treatment, Wnt signal sensor cells will be lysed with passive lysis buffer using recommended guidelines for the Dual Luciferase Assay kit (DLA) from Promega. According to DLA protocol, luciferase assay reagent (LAR) will be added to the lysate followed by Stop&Glo reagent to generate a luminescent signal. Luminescence will be measured with a 2-second premeasure delay followed by 10 second measurement period for each well. DLA assay includes built-in Renilla luminescence control to account for background signal. Based on previous experiments (Wilt *et.al.* 2010), we expect an exponential increase in luminescence with increasing concentration.

By coupling LiCl treatment with the siRNA treatment, we can demonstrate that knockdown of these genes lowers expression of β-catenin. In a 96-well culture plate $2x10^4$ cells will be seeded per well and then treated with siRNA. After siRNA treatment, media will be replaced with LiCl treatment. Following siRNA and LiCl treatment, RNA will be isolated via RNeasy Plus 96 kit and RT-qPCR will be performed with assays from QIAGEN targeting genes from Table 2 as well as β-catenin to verify that mRNA levels have been reduced. Assays will be run on an ABI Prism™ 7900HT Sequence Detection System qPCR machine using RT^2 SYBR® Green qPCR master-mix and standard protocol from QIAGEN. We expect the RT-qPCR assays to perform similar to those used determine siRNA efficiency and also expect a decrease in β-catenin levels.

Cell fractionation will separate proteins based on intracellular location permitting study of changes in localization of β-catenin due to altered Wnt/NFκB gene expression. Fractionation will be accomplished through use of Qproteome Cell Compartment kit from QIAGEN yielding four distinct protein fractions; cell membrane, cytosolic, nuclear, and cytoskeletal. This is accomplished through a sequence of four proprietary buffers which disrupts and solubilizes specific membranes to separate discrete fractions through centrifugation.

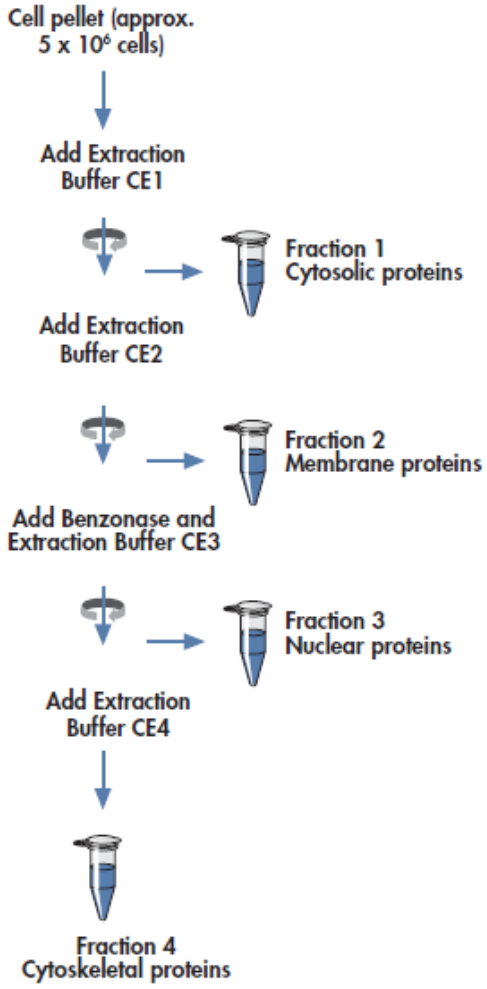

Figure 3: Overview of Qproteome kit workflow.

The plasma membrane will be disrupted first with Buffer 1 permitting isolation of cytosolic proteins in the supernatant after centrifugation. Resuspension of the first pellet in buffer 2 will solubilize the plasma membrane as well as membrane-bound organelles which will isolate cell membrane proteins in the supernatant after a second centrifugation step. Resuspension of the second pellet in buffer 3 will disrupt the nuclear membrane and solubilize nuclear proteins isolating nuclear fraction proteins in the supernatant with a third centrifugation.

Resuspension of the third pellet with buffer 4 will solubilize any remaining proteins, these mostly being cytoskeletal proteins. QIAGEN guidelines recommend at least $5x10^6$ cells be lysed.

Wnt signal sensor cells will be plated in triplicate 100mM dishes for each siRNA as listed in Table 2. Non-stimulated cells will serve as controls as listed in Table 4. After 24-hours growth, cells will undergo siRNA and LiCl treatment as previously described. After treatments, cells will be trypsinized and resuspended in Phosphate Buffered Saline (PBS). This single cell suspension will be counted and diluted to $5x10^6$ cells per pellet and then lysed according to QIAGEN protocol.

Table 4: Samples for Western Blot analysis

siRNA treated	**LiCl treated**	**Purpose**
(-)	(-)	Negative control
(-)	(+)	Positive control
(+)	(-)	Normalization
(+) from Table 2.	(+)	Test samples

Western Blot detection to determine altered protein level of target genes and localization of β-catenin.

These fractions will then be separated by SDS-page gel electrophoresis utilizing a pre-cast NuPage™ Bis-Tris gel at 8% polyacrylamide from ThermoFisher. Samples will be run through the gel at constant 150V for 90 minutes in 3-Morpholinopropane-1-sulfonic acid (MOPS) buffer in an XCell SureLock™ gel module after which the gel will then be transferred to a 0.45 μm pore nitrocellulose membrane to perform western blotting. Transfer of samples from gel to membrane will be performed at constant 30V for 2 hours in MOPS buffer in an XCell II™ Blot module. To limit background non-specific antibody binding, membrane blocking will be performed with 5% BSA solution in PBS with 0.1% Tween-20 for 1 hour at room temperature. Blocking solution will be removed and primary antibody added at 1:1000 dilution. Primary monoclonal antibodies

from AbCam listed in Table 5 to include no lysate well as negative control. Horseradish-peroxidase (HRP) conjugated secondary antibody will be utilized to quantify protein levels through chemi-luminescence. After siRNA and LiCl treatment, we expect target genes to have lower levels of protein and we expect β-catenin to be present in nuclear and cytoplasmic fractions.

Table 5: Primary monoclonal antibodies for western blot detection of protein levels.	
Ctnn-B	Gene of interest
AKT1	Gene of interest
FADD	Gene of interest
RELA	Gene of interest
TLR1	Gene of interest
TNFRSF10A	Gene of interest
ATP1A1	Membrane protein fraction positive control
HIST4h4	Nuclear protein fraction positive control
B-actin	Cytoskeletal protein fraction positive control
GAPDH	Cytoplasmic protein fraction positive control

Western blot antibody selection to control for fraction purity.

Time to complete: These experiments are estimated to take two years of study.

The results of Aim 1 will be analyzed and only those genes inducing a change in either β-catenin localization or protein level will be utilized in Aim 2.

Aim 2) **To demonstrate that lower cytoplasmic levels of β-catenin results in stimulation of EMT leading to an increase of cell motility.**

We hypothesize that loss of cytoplasmic β-catenin may result in stimulation of EMT as evidenced by an increase of cell motility. Transverse cell motility best mimics metastasis, and so Boyden chambers will be utilized to simulate escape of the basement membrane, specifically FluoroBlok™ 24-well basement membrane from Corning. The Boyden chamber will be inserted into the culture plate, and media added to ensure media in both lower and upper compartments. Following siRNA treatment, the Wnt signal sensor cells will then be chemically treated with TGFβ (Tian *et.al.* 2015; Kissin *et.al.* 2002) in order to stimulate EMT. Exogenously stimulating the siRNA treated cells will determine if alteration of β-catenin levels through Wnt/NFκB signaling can increase cell motility. Cells which have undergone EMT can be detected as motile cells present in the lower chamber via Calcien-AM fluorescence levels. Living cells will break down Calcien-AM generating a fluorescent signal while the Fluoroblok inserts have been developed by Corning to isolate light between the upper and lower chambers.

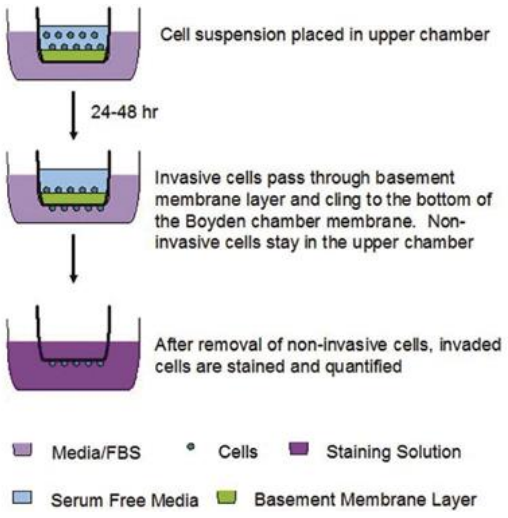

Figure 4. Operation of boyden chambers for motility assaying.

Used from https://www.cellbiolabs.com/cell-invasion-assays-24-well-basement%20membrane

Optimization of *TGFβ* treatment will be run on Wnt signal sensor cells without siRNA or LiCl treatment. In a 12-well plate, *TGFβ* and Calcien-AM will be added to the

lower chamber, a Fluoroblok Boyden chamber will be inserted into each well, and cells will be seeded at $2.5x10^4$ cells per well. Three replicates for each condition and three plates for each time period will be tested. Cells will be treated with 0 ng/mL, 5 ng/mL, 10 ng/mL, and 20 ng/mL over a course of 24 hours, 48 hours, 96 hours, and 172 hours. Fluorescence will be detected at 480nm/530nm. We expect a linear increase in number of cells in the lower chamber.

Given optimized *TGFβ* treatment, Wnt signal sensor cells will be treated with siRNA and then Wnt activity will be induced with LiCl as previously indicated. Cells will then be serum-starved to synchronize growth cycles. To induce EMT, first *TGFβ* treatment media will be added to the culture plate and Calcien-AM will be added to detect motility. Pre-prepared Fluoroblok Boyden chambers will be inserted to each well of 24-well plate and sensor cells will be seeded at $2.5x10^4$ cells per well, triplicate plates will be prepared. The treated cells will be allowed to grow based on findings of *TGFβ* treatment and fluorescence will be detected at 480nm/530nm based on length of *TGFβ* treatment.

Time to complete: These experiments are estimated to take one year of study.

REFERENCES

Abu-baker A, Laganiere J, Gaudet R, Rochefort D, Brais B, Neri C, Dion PA, Rouleau GA. 2013 Lithium Chloride attenuates cell death in oculopharyngeal muscular dystrophy by perturbing Wnt/β-catenin pathway. Cell Death and Disease. e821;doi: 10.1038/cddis.2013.342.

Cadigan K and Waterman M. 2012 TCF/LEFs and Wnt signaling in the Nucleus. Cold Spring Harb Perspect Biol. 4: a007906

Deng J, Miller S,Wang HY, Xia W, Wen Y, Zhou B, Li Y, Lin SW, Hung MC. 2002 β-catenin interacts with and inhibits NFκB in human colon and breast cancer. Cancer Cell. 2: 323-334.

Dinarello C. 1996 Biologic role for Interleukin-1 in Disease. Blood. 87(8): 2095-2147.

Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A. 2003 Phosphorylation by glycogen synthase kinase-3β down-regulates Notch activity, a link for Notch and Wnt pathways. Journal Biological Chemistry. 278(34): 32227-32235.

Finn OJ. 2012 Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. Annals of Oncology. viii6-viii9.

Gordon K, Dong M, Chislock E, Fields T, Blobe G. 2008 Loss of type III transforming growth factor β receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition during pancreatic cancer progression. Carcinogenesis. 29(2): 252-262.

Hanahan D and Weinberg RA. 2000 The hallmarks of cancer. Cell Vol. 100: 57-70.

Hayden MS and Ghosh S. 2011 NFκB in immunobiology. Cell Research. 223-244.

Hinz S, Pagreols-Raluy L, *et.al.* 2007 Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res. 67: 8344-8350.

Howard S. Deroo, T. Fujita Y., Itasaki N. 2011 A positive role of Cadherin in Wnt/βcatenin signaling during Epithelial-Mesenchymal Transition. PLoS One. 6(8): e23899.

Kaler P, Godasi B, Augenlicht L, Klampfer L. 2009 The NFκB/AKT-dependent induction of Wnt signaling in colon cancer cells by macrophages and IL-1β. Cancer Microenvironment. 2: 69-80.

Kam Y and Quaranta V. 2009 Cadherin-bound β-catenin feeds into the Wnt pathway upon Adherens Junctions dissociation: Evidence for an Intersection between β-catenin pools. PloS One. 4(2): e4580.

Katoh M and Katoh M. 2009 Transcriptional mechanisms of Wnt5a based on NFκB, Hedgehog, TGFβ, and Notch signaling cascades. International Journal of Molecular Medicine. 23: 763-769.

Khanbolooki S, Nawrocki S, Arumugam T, Andtbacka R, Pino M, Kurzrock R, Logsdon C, Abbruzzese J, McConkey D. 2006 Nuclear factor-κB maintains TRAIL resistance in human pancreatic cancer cells. Mol Cancer Ther. 5(9): 2251-2260

Kissin E, Lemaire R, Korn J, Lafyatis R. 2002 Transforming Growth Factor β induces fibroblast Fibrillin -1 matrix formation. Arthritis & Rheumatism. 46(11): 3000-3009.

Krelin Y, Voronov E, *et.al.* 2007 Interleukin-1β-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. Cancer Res. 67(3): 1062-1071

Lamouille S, Xu J, Derynck R. 2014 Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 178-196.

Linde N, Casanova-Acebes M, *et.al.* 2018 Macrophages orchestrate breast cancer early dissemination and metastasis. Nature Communications. 9:21

Liou H. 2002 Regulation of the immune system by NFκB and IκB. Journal of Biochemistry and Molecular Biology. 35(6): 537-546.

Lu M, Marsters S, Ye X, Luis E, Gonzalez L, Ashkenazi A. 2014 E-Cadherin couples death receptors to the cytoskeleton to regulate apoptosis. Molecular Cell. 54: 987-998

Ma B, Hottiger MO. 2016 Crosstalk between Wnt/β-catenin and NF-κB signaling pathway during inflammation. Front. Immunol. 7: 378

MacDonald B, He X. 2012 Frizzled and LRP5/6 Receptors for Wnt/β-catenin signaling. Cold Spring Harb Perspect Biol. 4: a007880.

Naskar D, Maiti G, Chakraborty A, Roy A, Chattopadhyay D, Sen M. 2014 Wnt5a-Rac1-NFκB homeostatic circuitry sustains innate immune functions in macrophages. J Immunol. 192: 4386-4397.

Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. 1999 E-cadherin binding prevents β-catenin nuclear localization and β-catenin/LEF-1-mediated transactivation. Journal of Cell Science. 112: 1237-1245.

Pai SG, Carniero BA, Mota JM, Costa R, Leite CA, Barrossa-Sousa R, Kaplan JB, Chae YW, Giles FJ. 2017 Wnt/beta-catenin pathway: modulating anticancer immune response. Journal of Hematology & Oncology. 10: 101

Palacios F, Tushir JS, Fujita Y, D'Souza-Shorey C. 2005 Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. Mol. Cell Biol. 25(1): 389-402

Pećina-Šlaus N. 2010 Wnt signal transduction pathway and apoptosis: a review. Cancer Cell International. 10: 22.

Pomerantz J and Baltimore D. 2002 Two pathways to NF-kappa-B. Molecular Cell. 10: 693-701

Rajasekhar V, Studer L, Gerald W, Socci N, Scher H. 2011 Tumour-initiating stem-like cells in human prostate cancer exhibit increased NFκB signaling. Nature Communications. 2: 162.

Rao TP and Köhl M. 2010 An updated overview on Wnt signaling pathways: A prelude for more. Circ Res. 106: 1798-1806.

Roy P, Mukherjee T, Chatterjee B, Vijayaragavan B, Banoth B, Basak S. 2017 Non-canonical NFκB mutations reinforce pro-survival TNF respsonse in multiple myeloma through an autoregulatory RelB:p50 NFκB pathway. Oncogene. 36: 1417-1429.

SEER Cancer Statistics accessed 12/16/2015 Factsheets: Female Breast Cancer. National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/statfacts/html/breast.html.

Spriggs M, Lioubin P, Slack J, Dower S, Jonas U, Cosman D, Sims J, Bauer J. 1990 Induction of Interleukin-1 receptor (IL-1R) on monocytic cells. Journal Biological Chemistry. 265(36): 22499-22505.

Sulzer M, Leers M, van Noord J, Bollen E, Theunissen P. 1998 Reduced E-cadherin expression is associated with increased lymph node metastasis and unfavorable prognosis in non-small cell lung cancer. Am J Respir Crit Care Med. 157: 1319-1323.

Sun J, Hobert M, Duan Y, Rao A, He T, Chang E, Madara J. 2005 Crosstalk between NFκB and β-catenin pathways in bacterial-colonized intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol. 289: 129-137.

Sun S. 2012 The noncanonical NFκB pathway. Immunol Rev. 246(1): 125-140.

Terry S, Savagner P, Ortiz-Cuaran S, Mahjoubi L, Saintigny P, Thiery JP, Chouaib S. 2017 New insights into the rol of EMT in tumor immune escape. Molecular Oncology. 11: 824-846

Tian B, Li X. *et.al.* 2015 Analysis of the TGFβ induced program in primary airway epithelial cells shows essential role of NFκB/RelA signaling network in type II epithelial mesenchymal transition. BMC Genomics. 16: 529

Valkenberg K, Graveel C, Zylatra-Diegel C, Zhong Z, Williams B. 2011 Wnt/β-catenin Signaling in normal and cancer stem cells. Cancers. 3: 2050-2079.

Weeraratna A, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent J. 2002 Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell. (1): 279-288.

Willert K, Nusse R. 2012 Wnt Proteins. Cold Spring Harb Perspect Biol. 4: a007864.

Wilt G, Jiang Q, and Devgan V. 2010 Cross-regulation of Wnt signaling by NFκB pathway components. American Society Cell Biology Signal Transduction I program 881, poster B1338.

Zheng X, Xiang Z, *et.al.* 2011 Differential expression of Wnts, β-catenin and E-cadherin in hEFs and normal, abnormal karyotype hES cells during culture in vitro. Journal of Life Sciences. 5: 483-387