Regulation of cadherin-11 by GSK3 and TGFβ1 in cancer cells

A Dissertation
Submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Cell Biology

By

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Washington, DC
March 17, 2008
DEDICATION

This dissertation is dedicated to my family for their constant love and support and to my loving husband without whom I am nothing.
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ABSTRACT

Cadherin-11 is a cell-cell adhesion molecule important during embryogenesis as well as bone morphogenesis. Exogenous cadherin-11 expression has been shown to increase cell invasion and metastasis in breast cancer cells. Here I show that cell density, GSK3 inhibition, and TGFβ1 pathway activation regulate cadherin-11 expression in breast and prostate cancer cells. Density has been shown to affect cadherin expression in various cell types. I found that cadherin-11 RNA and protein expression increase with increasing cell density. Further, treatment with GSK3 inhibitors, LiCl or BIO, greatly reduces both the basal level and density-dependent increase in cadherin-11 expression. Using anti-β-catenin RNAi, I found that GSK3 inhibition has two mechanisms of regulating cadherin-11 expression. The first is β-catenin-independent and is important to the regulation of both cadherin-11 RNA and protein. The second mechanism is β-catenin-dependent and regulates cadherin-11 protein expression. Actinomycin D and RNA polymerase II chromatin immunoprecipitation (ChIP) analysis reveal a post-transcriptional mechanism of regulation. Investigation of the cadherin-11 3’-untranslated region (UTR) uncovers several consensus sequences important to RNA stability or translational regulation. GSK3 inhibition utilizes several mechanisms to regulate cadherin-11 expression.
TGFβ1 treatment also acts to regulate cadherin-11 expression by repression. TGFβ1 reduces basal levels of cadherin-11 and attenuates the effect of density. The mechanism of TGFβ1 regulation is Smad-independent, although the signal is undoubtedly transmitted through the TGFβ type I receptor (TβRI). A TβRI kinase inhibitor alleviates TGFβ1-dependent repression of cadherin-11 and has a subtle effect on endogenous cadherin-11. Investigation into other Smad-independent pathways activated by TGFβ1 binding suggests that p38 MAPK most likely plays a role in cadherin-11 regulation. ChIP analysis of samples treated with TGFβ1 reveals that cadherin-11 is not transcriptionally downregulated again pointing to RNA stability as the primary mechanism of cadherin-11 regulation.

To conclude, cadherin-11 is regulated by GSK3 inhibition and TGFβ1, but not through the well-known mechanisms of these pathways. Further investigation is needed to determine how exactly these pathways affect cadherin-11 expression through RNA stability. Considering the possible interacting proteins, I suggest the exploration of RNA binding proteins such as HuR, β-catenin, and Puf proteins.
LIST OF ABBREVIATIONS

ABSTRACT:

GSK3 – Glycogen synthtase kinase 3
TGFβ1 – Transforming growth factor β1
LiCl – Lithium chloride
BIO – (2‘Z,3‘E)-6-Bromoindirubin-3’-oxime
RNAi – RNA interference
ChIP – Chromatin immunoprecipitation
UTR – Untranslated region
TβRI – TGFβ type I receptor

CHAPTER I:

FLS – Fibroblast-like synoviocyte
Gln – Glutamine
Ala – Alanine
Val – Valine
His – Histidine
APC – Adenomatous polyposis coli
ARE – Adenine-uridine rich element
AUF1 – ARE/poly-U binding degradation factor 1
TTP – Tristetraprolin
KSRP – K homology-type splicing regulatory protein
TIA-1 – T-cell restricted intracellular antigen
TIAR – TIA-related protein
Hsp – Heat-shock protein
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
PUF – Pumilio/FBF
CPEB – Cytoplasmic polyadenylation element binding protein
miRNA – MicroRNA
siRNA – Small-interfering RNA
pri-miRNA – Primary miRNA
RISC – RNA-induced silencing complex
CK1 – Casein kinase 1
TCF – T cell factor
LEF – Lymphoid exchange factor
Ago – Argonaute
CHAPTER II:

OB-cadherin – Osteoblast-cadherin
Ser – Serine
NaCl – Sodium chloride
meBIO - 1-Methyl-BIO
WT – Wild type
DN – Dominant negative
DMEM – Dulbecco’s Modified Eagle’s Medium
FBS – Fetal bovine serum
PCR – Polymerase chain reaction
RT-PCR – Reverse transcription-PCR
CDH11 – cadherin-11
eIF6 – Eukaryotic translation initiation factor 6
PI3K – phosphatidylinositol 3-kinase
MDA231 – MDA-MB-231
RNA Pol II – RNA polymerase II
ActD – Actinomycin D

CHAPTER III:

EMT – Epithelial-to-mesenchymal transition
PAI-1 – Plasminogen activator inhibitor 1
MMP – Matrix metalloproteinase
TIMP – Tissue inhibitor of metalloproteinase
VEGF – Vascular endothelial growth factor
SEM – Standard error of the mean

CHAPTER IV:

CPE – Cytoplasmic polyadenylation element
PBE – Pumilio-binding element
P-body – Processing Body
I would like to thank Dr. Stephen Byers who served as my mentor throughout my PhD. I am grateful for his scientific expertise, the intellectual freedom he allowed, and his guidance. I would also like to thank Dr. Robert Lechleider who served as a co-mentor during the initial years of my PhD. He guided and supported me through my inexperience with both laboratory techniques and writing. He is a great scientist, and I will always value his opinion. To my labmates, past and present, I thank you for your sincerity, patience, and scientific expertise. I thank my thesis committee members, past and present, Dr. Ian Gallicano, Dr. Robert Lechleider, Dr. John Casey, Dr. Partha Banarjee, Dr. Lopa Mishra, and Dr. Christopher Taylor, for all of their direction that has helped me to become a better scientist. Specifically, I thank Dr. Ian Gallicano who never flinched at my constant interruptions and requests for help and advice.

I have been lucky enough to have been a part of a lab full of friends. We have seen each other grow as people as well as scientists. I thank them for always having an open ear and words of advice and encouragement. They have all played a valuable part in my education, and I am a better scientist and person for having worked with them. I especially want to thank Marcy Beildeck, for her friendship and for opening her home to me over the past months.

I want to thank my family. They fostered an early interest in science and supported me through years of rock collections, microscopes, and chemistry sets.
Finally, I want to thank my husband, Bobby. I am eternally grateful for his constant support through years of living in different cities; he is always there for me. I love you.
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INTRODUCTION

A few questions are at the forefront of metastasis gene research. What genetic changes are necessary and sufficient for cells to become metastatic? Do cancer cells that will metastasize, or already have metastasized, express any biomarkers that will distinguish them from non-metastatic cells? Are there metastasis genes which function in a limited set of tumor types? Ultimately, the answers to these basic questions will be applied to benefit patients. For example, genetic markers could be used to diagnose tumor type, grade, and perhaps even disease stage, including prediction of the location of possible metastases. Likewise, gene therapies may be an alternative to chemotherapy for treating some tumors. Thus, the newly emerging field of metastasis genetics offers many possibilities for future innovations in the treatment of patients with cancer.

In 2007, breast cancer was expected to be the second leading cause of cancer-related deaths in women and to be the most new cancer cases in women; the statistics are equivalent for men and prostate cancer (50). Women over 70 with recurring breast cancer have a 1:15 chance of developing metastatic disease (50). The odds are even worse for men; the risk of developing invasive prostate cancer is 1:7 in men over 70 (50). Of cancers that will metastasize, breast and prostate are the most likely cancers to metastasize to the bone. Scientists have studied cancer cells from these patients hoping to determine how they differ from cells that do not prefer bone metastasis.

The majority of patients dying from cancer of the breast or prostate have metastases to the skeleton. Although bone metastases are incurable, patients survive
several years suffering serious morbidity, including fractures, spinal cord compression, severe bone pain, and hypercalcemia (11, 37). Bone is a dynamic tissue that undergoes constant remodeling in order to maintain skeletal strength (Figure 1). When bone is broken down growth factors from the mineralized bone matrix are released into the microenvironment, providing fertile soil for the growth of metastatic tumor cells. These growth factors can influence the phenotype of tumor cells to favor their growth in bone. Tumor cells, in turn, secrete additional factors that act on bone cells, causing skeletal responses that characterize bony metastasis. These local interactions between tumor cells and bone form a vicious cycle, which underlies the development of skeletal metastases (Figure 1). The unique properties of the bone microenvironment and the local interactions between tumor and bone cells that favor metastasis are just now being clarified (11, 37, 39). The work described in this thesis investigates one of these factors, the cell adhesion molecule, cadherin-11.

Another disease of the bone and joints, rheumatoid arthritis, has been described as having characteristics similar to cancer (30). Thought to be a disease caused by an autoimmune response and excessive inflammation, investigators are helping us to understand other possible causes of rheumatoid arthritis. Many of the cytokines that promote cancer progression are also found in rheumatoid joints. The synovial lining contains mesenchymal cells called fibroblast-like synoviocytes (FLSs) that invade the joint destroying cartilage and bone in the process. As the cells of the synovium invade the joint, degrading bone and cartilage, growth factors are released promoting growth
and invasion in a manner similar to bone metastasis. Fortunately, unlike cancer cells, FLS cells do not form metastases. As in bone homing cancer cells, cadherin-11 is expressed in the synovium and may constitute a promising therapeutic target.

Many researchers believe that a disruption of the fine-tuned balance of adhesion and detachment can alter properties of cell motility and promote the transition from normal to diseased states, such as cancer or arthritis. If cells are either nonadherent or too adherent, that is, the surface upon which they intend to move is too slick or too sticky, cells cannot move adequately. Thus, cell adhesion molecules have roles in both promoting and inhibiting metastasis. Specific families of adhesion molecules whose expression correlates with metastasis include selectins, integrins, and cadherins (85).

Cells expressing the highest levels of cadherin-11 are frequently metastatic breast and prostate tumor cells that have migrated to the bone. Based on this fact, my work investigates the mechanisms of cadherin-11 regulation using breast and prostate cancer cell model systems. Cadherin-11 is expressed in several highly metastatic tumor cell lines and the fibroblast-like synoviocyte (FLS) cells of the synovium (94, 122). In my work, the regulatory mechanisms of cadherin-11 gene expression at both the transcriptional and post-transcriptional levels have been studied specifically with respect to GSK3β inhibition and TGFβ1 activation.
CADHERIN-11

Cadherin-11, also known as osteoblast-cadherin, was first discovered in mouse osteoblasts, and cells of osteo-lineage are still thought to be the primary cadherin-11-expressing cell type in the normal adult organism. During development, cadherin-11 is expressed by cells of mesodermal origin with a mesenchymal phenotype especially cells of the developing kidney and brain (113). In 2004, Valencia et al. discovered that fibroblast-like synociocytes (FLSs) of the joint express cadherin-11 (122). It seems that cadherin-11 expression is necessary for the FLS cells to align on the surface of the synovium (Figure 2); if blocked or replaced with another cadherin, cells no longer resemble a wild type synovium (57). Symptoms of rheumatoid arthritis include excessive inflammation, the cause of bone and cartilage damage. The synovium becomes infiltrated with leukocytes and a pannus, or extension of the synovium, forms (Figure 2C) (73). When cadherin-11 function is blocked the pannus fails to form (Figure 2D) (63). In the absence of pannus formation, the arthritic joint sustains damage to the bone, but the cartilage is no longer affected. This work forecasts a promising future for interest in cadherin-11 research.

Cadherins are a family of transmembrane glycoproteins that mediate Ca\(^{2+}\)-dependent homophilic cell-cell adhesion. All cadherins have five extracellular, cadherin domains, a transmembrane domain, and an intracellular domain that is able to interact with intracellular proteins (Figure 3A). Cadherin-11 is type II classical cadherin having a QAV (gln-ala-val) cell adhesion recognition sequence instead of the
type I cadherin-specific HAV (his-ala-val) sequence (48, 110, 116, 118). Among cadherin family members, cadherin-11 is unique because it has two known isoforms (Figure 3) (54, 94). The first isoform, which will hereafter be referred to as cadherin-11, has a protein structure typical of most type II cadherins. The intracellular domain of cadherin-11 interacts with β-catenin and other intracellular proteins, such as p120 and α-catenin, important to signaling and motility (55). The second isoform, cadherin-11 variant, contains the same extracellular cadherin domains but has an alternate splice site between exons 11 and 12 resulting in inclusion of 179 bp of unique intronic sequence (Figure 3B). This splicing event results in a frameshift, truncating the transmembrane domain (Figure 3A). The catenin binding is replaced by an intracellular domain with a new exon which exhibits homology to tyrosine kinases eliminating contact with intracellular catenins but perhaps promoting contact with a yet unknown molecule (28, 54).

Cadherin-11 also plays a role in cancer progression and metastasis including breast, prostate, and colon cancer (7, 84, 94, 120). Cadherin-11-expressing breast and prostate cancers are categorized as the most aggressive tumors, metastasizing to distant organs including the bone. One theory suggests that tumor cells expressing cadherin-11 activate bone remodeling osteoclasts or osteoblasts leading to a vicious cycle of bone resorption (11, 37, 99). Previously published studies suggest that the expression of cadherin-11 variant together with cadherin-11 greatly increases a cell’s invasive
potential (28). Thus, cadherin-11 and cadherin-11 variant play an important role in the invasive potential of breast cancer.

I.B. REGULATION OF CADHERIN-11 EXPRESSION

Regulation of cadherin-11 in normal, cancerous, and rheumatoid arthritic tissues and cells remains somewhat enigmatic; one area of comparison to other cadherins is with respect to cell confluency. Dense cell cultures resemble a differentiated epithelium with high levels of cadherin and beta-catenin primarily located in adherens junctions. Previously published data show that with increased cell density, N-cadherin protein levels decrease in rat Schwann cells, suggesting that N-cadherin plays a pertinent role in adhesion in mesenchymal cells (126). In contrast, it has been reported that E-cadherin expression levels are increased by cell density in a colon cancer cell line (13). At sparse densities, E-cadherin levels were low leading to increased cytoplasmic and nuclear β-catenin and upregulating Slug expression, a known E-cadherin transcriptional repressor. This in turn leads to further downregulation of E-cadherin expression. Thus, it appears that regulation of both N- and E-cadherin by manipulating cell density is important in tumorigenesis.

The focus of this work has been to examine cadherin-11 regulation in response to cytokines, such as Wnt and TGFβ1, released as a by-product of bone remodeling, and therefore has focused on breast and prostate cancer cells derived from metastases as model systems.
I.C. WNT SIGNALING AND GSK3 INHIBITION

β-catenin is a ubiquitously expressed protein with a mounting number of roles in the cell. Two of its best known functions are at adherens junctions and as a signaling intermediate in the Wnt pathway. Canonical Wnt signaling plays an important role in many cellular functions, such as cell proliferation and differentiation. Aberrant activation of canonical Wnt signaling is involved in various human malignancies preventing β-catenin degradation leading to unregulated transcription (96).

In the absence of canonical Wnts, GSK3 binds and phosphorylates β-catenin, in a multi-protein complex including axin, and adenomatous polyposis coli (APC). Phosphorylation of β-catenin results in β-catenin degradation via the ubiquitin-proteosome pathway (61, 76). In the presence of canonical Wnts, Disheveled prevents GSK3-dependent phosphorylation of β-catenin resulting in its accumulation in the cytoplasm (96) (Figure 4). β-catenin is then able to translocate to the nucleus where it complexes with members of the TCF/LEF family of transcription factors upregulating or activating target genes (2, 6, 33, 34, 44). Once in the nucleus, the β-catenin/LEF/TCF complex activates the transcription of many target genes, such as c-myc and cyclin D1 (35, 41, 112, 119). In a transformed cell, β-catenin may be stabilized due to either mutations in APC (83, 101, 115) or β-catenin itself preventing phosphorylation by GSK3 (60, 81, 101).

Having at least 50 known substrates, in addition to β-catenin, the activity of GSK3 must be tightly regulated. GSK3 function can be regulated by four distinct
mechanisms: phosphorylation of GSK3 itself, phosphorylation of its substrates, protein complex association, and subcellular localization. Regulation of GSK3 by Akt is the best-defined mechanism of GSK3 inhibition. Activated Akt phosphorylates GSK3β on serine 9 and GSK3α on serine 21 inhibiting GSK3 activity. Secondly, GSK3 is known as a discriminating kinase that will only bind to and phosphorylate its target proteins once they have been primed by a previous phosphorylation event (125). β-catenin is a well-studied example of how primed phosphorylation is necessary for GSK3 binding. Thirdly, GSK3 functions as part of complexes such as the Axin-APC-GSK3 complex. If this complex is unable to form due to mutations in any of the proteins, GSK3 is no longer able to bind its substrate, in this case, β-catenin. Finally, cellular localization of GSK3 can alter its function due to compartmentalization (25).

When GSK3 regulation fails, the development of disease frequently ensues. Because of the role GSK3 plays in the insulin pathway, GSK3 inhibitors may soon be used to treat diabetes. Studies of inflammatory diseases such as arthritis have found that GSK3 function is important in inflammation (43).

The relationship between cadherin-11 and Wnt signaling remains poorly defined. In the developing Xenopus embryo cadherin-11 is expressed in cranial neural crest cells and animal cap cells (123). Furthermore, XCadherin-11 RNA is increased by XWnt-8 overexpression (40). Cadherin-11 expression with respect to Wnt family members continues to be an ill-defined field.
I.D. TRANSFORMING GROWTH FACTOR-β (TGFβ)

TGFβ family members play a role in development as well as cancer progression (17, 117). TGFβ1 begins the process of signal transduction by binding to the TGFβ type II receptor (Figure 5). Once ligand bound, the type II receptor, a serine/threonine kinase, phosphorylates and activates the TGFβ type I receptor (77). This receptor then phosphorylates and activates Smads2 and 3. The activated Smads along with Smad4, the co-Smad, translocate to the nucleus synergistically binding DNA in order to regulate target gene transcription (77). TGFβ1 binding also activates the p38 MAPK, Erk, JNK, and S6 kinase pathways independently of Smad signaling (16, 17, 67).

During the early stages of carcinogenesis, TGFβ acts as a tumor suppressor, but in late stage carcinogenesis, TGFβ takes on a tumor-promoting role. Extensive studies have shown that TGFβ family members cause growth arrest in early stage tumor cell lines, but can enhance motility and invasiveness in late-staged disease (17). TGFβ1 is widely expressed in bone tissue and is released into the extracellular matrix as bone is reabsorbed, making bone a favorable environment for metastatic tumor cell growth (37). Due to its close association with bone, TGFβ1 also plays a role in joint inflammation and arthritis (5, 95). A microarray of the synovium from osteoarthritic and rheumatoid arthritic tissue showed changes in TGFβ1 pathway members including TGFβ1 itself (5, 95).

Little is known about the relationship between TGFβ1 and cadherin-11. Cadherin-11 expression increased in both extravillous cytotrophoblast cells and human
tubular epithelia cells of the kidney in response to TGFβ1 (31, 32). Outside of these two reports, the way in which these two molecules interact is not well-defined. Both cadherin-11 and TGFβ1 are prominently expressed in cells of the bone and joint. Therefore it is possible that they may both function at this interface.

I.E. POST-TRANSCRIPTIONAL REGULATION

My work ultimately draws the conclusion that cadherin-11 is regulated by a post-transcriptional mechanism. Post-transcriptional regulation affects either RNA stability or translation initiation. Shaw-Kamens sequences, also known as adenine-uridine rich elements (AREs), are known to be involved in this process (109). The list of proteins that bind to this sequence includes Hu protein family members (HuR, HuB, HuC, HuD), ARE/poly-U binding degradation factor (AUF) 1, tristetraprolin (TTP), the K homology-type splicing regulatory protein (KSRP), T-cell restricted intracellular antigen (TIA)-1, TIA-related protein (TIAR), heat shock protein 70 (Hsp70), β-catenin, and GAPDH (reviewed in (24)). Many other proteins are known to bind to specific sequences and affect the mRNA translational rate including cytoplasmic polyadenylation element binding protein (CPEB) and PUF family members such as Pumilio. Recently Pique et al reported that the combination of PUF and CPEB binding sites determines whether an mRNA will be translated (93). Finally, microRNA (miRNA) binding also affects mRNA translation. miRNA: mRNA sequence complementarity determines translational processing. This process is described in
Various mechanisms are known to regulate mRNAs post-transcriptionally and pre-translationally. Each has a specific manner of regulating expression, all exploiting the 3’-untranslated region (UTR) in their processing.

The term RNA interference, or RNAi, was coined by Craig Mellow and Andrew Fire who reported that double-stranded RNA dramatically silenced the *C. elegans* unc-22 gene; a result not seen with mRNA or anti-sense RNA injections (29). Since that time, investigators have been using double-stranded RNA to knock-down protein expression in a variety of organisms. Small-interfering RNA (siRNA) was the basis for the work of Mellow and Fire and continues to be a scientific staple. This mechanism of regulation requires a 19-22 double-stranded oligonucleotide that binds a specific complementary sequence in the mRNA leading to message degradation. This technique has been successfully used to knock-down genes in many model systems.

The focus of RNAi has turned to endogenous siRNA-like oligonucleotides called microRNAs (miRNAs). Although first described in 1991, miRNA has only recently become a popularly investigated mechanism of regulation (68). miRNAs have a 5’ core binding region of six nucleotides, known as the seed sequence, and little complementarity between the 3’ end of the miRNA and the target mRNA. Minimal complementarity between the miRNA and mRNA allows many miRNAs to bind to one mRNA and one miRNA to bind to many mRNAs (71, 72). The stability of the base-pairing in the 5’ end determines the type of repression by the miRNA. miRNAs with a high degree of complementarity, typical of plant miRNAs, tend to be degraded like
siRNA-mediated downregulation (46). Most animal miRNAs have low complementarity outside of the seed sequence leading to sequestration into processing bodies, or P-bodies, and translational repression with no mRNA degradation (19, 20).

Primary miRNA, pri-miRNA, is transcribed from the genome either as a single message, a poly-cistronic message, or as spliced intronic sequence. Pri-miRNA is then processed by Drosha into a double stranded hairpin called pre-miRNA and exported from the nucleus (69, 74, 129). In the cytoplasm, Dicer cleaves the hairpin RNA into mature double-stranded miRNA (Figure 6) (36, 45, 56, 58, 70). Then one strand of the double-stranded miRNA is loaded into the RNA-induced silencing complex (RISC) (107). The miRNA in the RISC complex then guides the process of RNA silencing (Figure 6). The RISC complex is composed of several proteins, one of which is Argonaute. Although four Argonaute isoforms exist, only Argonaute 2 has RNase activity and can cleave the target mRNA (79). As mentioned previously, the degree of complementarity between the miRNA and mRNA determines the fate of the complex.

The discovery of miRNA has had a major impact on many areas of research including disease research. miRNAs present a new mechanism by which cells are regulated and also misregulated. Many miRNAs have been found during various stages of development and are important to the viability of the embryo. The role of miRNA in the heart is well documented as it relates to both development and disease (62). Not surprisingly, investigators have found misregulated miRNAs in cancers from the colon.
to the brain (8, 106). Within a relatively short period of time, the interest in miRNAs and their potential for both causing and curing disease has been colossal.

I.F. STATEMENT OF PURPOSE

Cadherin-11 plays a role in development, but the knockout mouse is viable. These animals have reduced bone density and a reduction in the calcification of the frontal suture (52). Despite having a redundant role in development, cadherin-11 is clearly important in cancer progression. Tumors expressing cadherin-11 are more likely to metastasize resulting in tumor infiltration of the entire organism. For this reason, cancer cells were used as a model system for these studies. I hoped cells aberrantly expressing cadherin-11 would allow me to uncover the mechanisms regulating cadherin-11, specifically in cancer. Cadherin-11 was more recently found to be expressed in the synovial lining and to play a role in the development of rheumatoid arthritis (57, 63, 122). Because of the large patient population affected by rheumatoid arthritis, this model system will most likely play a role in the future of cadherin-11 research.

In cancer and rheumatoid arthritis, cadherin-11 expression promotes disease, therefore its downregulation may prevent disease progression. For this reason I set out to find the mechanism behind cadherin-11 regulation. There are many available therapies that may have either a positive or negative impact on cadherin-11, and knowing how cadherin-11 will respond allows physicians to target a patient’s therapy.
Cell survival requires a perfect balance of conditions; in the event these conditions change and go awry the cells will either die or become malignant. Cancer development and progression involves multiple pathways any of which may be responsible for the upregulation of cadherin-11. Two pathways often misregulated during cancer development and progression are Wnt and TGFβ1 signaling. Early reports concerning cadherin-11 regulation found that cadherin-11 was not expressed in the absence of XWnt and that cadherin-11 expression increased in the presence of TGFβ1 (32, 40). Preliminary experiments using TGFβ1 or LiCl affected cadherin-11 expression in cancer cells suggesting they are important factors in cadherin-11 regulation. Therefore in the work described in this dissertation I set out to determine the signaling molecules regulating cadherin-11 including: 1) the canonical Wnt family, specifically through GSK3 inhibition and 2) TGFβ1 signaling proteins
I.G. Figure Legends

Figure 1. A schematic diagram of the bone remodeling cycle.
Adapted from Koichi Matsuo’s diagram found on
http://www.coe-stemcell.keio.ac.jp/member/matsuo.html

Figure 2. Cadherin-11 and arthritic inflammation.
This schematic depiction, adapted from (73), depicts arthritis development in the presence and absence of cadherin-11.

Figure 3. A schematic depiction of cadherin-11 function, splicing, and isoforms.
This diagram highlights the interaction of cadherin-11 with intracellular proteins.

Figure 4. Wnt signaling pathway.
This schematic diagram, taken from (86), illustrates the Wnt pathway in the absence and presence of ligand.

Figure 5. TGFβ1 signaling pathway.
This schematic diagram, taken from (17), illustrates the Smad-dependent and Smad-independent pathways of TGFβ1 signaling.
FIGURE 6. miRNA PROCESSING.

This schematic diagram, taken from (105), illustrates miRNAs processing from transcription to cleavage.
FIGURE 1. A SCHEMATIC DIAGRAM OF THE BONE REMODELING CYCLE
Adapted from Koichi Matsuo’s diagram found on
http://www.coe-stemcell.keio.ac.jp/member/matsuo.html
Figure 2. Cadherin-11 and arthritic inflammation
FIGURE 3. A schematic depiction of cadherin-11 function, splicing, and isoforms

A

Cadherin-11

Cadherin-11 variant

B

EXON 11

179bp

EXON 12

Variant

Wild type
FIGURE 4. THE WNT SIGNALING PATHWAY
From Nemeth and Bodine. Cell Res. 2007 Sept; 17(9): 746-58. (86)
FIGURE 5. THE TGFβ1 SIGNALING PATHWAY
FIGURE 6. miRNA PROCESSING
From Saumet and Lecellier. Retrovirology. 2006 Jan 12;3:3. (105)
CHAPTER II
THE WORK PRESENTED IN CHAPTER II WILL BE PUBLISHED AS FOLLOWS:

FARINA AK, FELTES CM, BYERS SW. GSK3β AND β-CATENIN REGULATION OF CADHERIN-11 EXPRESSION IN PROSTATE AND BREAST CANCER CELLS. MOLECULAR AND CELLULAR BIOLOGY. IN PRESS.

II.A. ABSTRACT

The cell-cell adhesion molecule cadherin-11 is important in embryogenesis and bone morphogenesis, invasion of cancer cells, lymphangiogenesis, homing of cancer cells to bone, and rheumatoid arthritis. However, very little is known about the regulation of cadherin-11 expression. Here I show that cell density and inhibition of GSK-3β regulate cadherin-11, but not N-cadherin expression in cancer cells. Inactivation of GSK3β with LiCl or BIO, a GSK3 inhibitor, and GSK3β knockdown with siRNA markedly repressed cadherin-11 mRNA and protein levels. Two targets of GSK3, β-catenin and Snail, although clearly regulated by LiCl are not important for regulation of cadherin-11 mRNA. RNA Polymerase II chromatin immunoprecipitation experiments showed that inhibition of GSK3 does not affect transcription of the cadherin-11 gene. Although the cadherin-11 3’-UTR contains regulatory elements and putative microRNA target sites and is subject to regulation by Dicer, its activity is not regulated by GSK3 inhibition or density. However the increase in β-catenin that results from inhibition of GSK3 is important in the regulation of cadherin-11 protein but not mRNA. These data indicate that GSK3 regulates cadherin-11 expression in two ways: first a β-catenin-independent mechanism of cadherin-11 mRNA regulation, and second a β-catenin-dependent mechanism that effects on cadherin-11 protein levels.
II.B. INTRODUCTION

Cadherin-11 was first identified in mouse osteoblasts and is normally expressed in cells with a mesenchymal phenotype and of mesodermal origin, including the mesenchyme of the kidney and brain during development (113). Cadherin-11 is also expressed in synoviocytes and is important for the formation of a pannus, characteristic of rheumatoid arthritis (57, 63). The cadherin-11 knockout mouse exhibits bone and behavioral abnormalities and is resistant to induction of rheumatoid arthritis (52, 63). Finally, cadherin-11 is expressed in several types of cancer including breast and prostate cancers, osteocarcoma, and colon cancer; cadherin-11 expression is associated with the most aggressive and most metastatic cancer cells (7, 37, 39, 94, 120).

Little is known about the regulation of cadherin-11 expression. TGFβ1 regulates cadherin-11 expression in cultured extravillous cytotrophoblasts, and progesterone, but not 17-beta-estradiol, regulates cadherin-11 in cultured endometrial stromal cells undergoing decidualization (9, 32). During development cadherin-11 is expressed in cranial neural crest cells of the developing Xenopus embryo and in Xenopus animal cap cells; XWnt-8 overexpression increases Xcadherin-11 mRNA (40, 123).

The principle pathway associated with canonical Wnt signaling involves inhibition of GSK3. GSK3 is intricately involved in many pathways essential to cellular function. In the absence of canonical Wnts, GSK3 binds and phosphorylates β-catenin, in a multi-protein complex. GSK3-dependent phosphorylation results in β-catenin
protein degradation (61, 76). In addition to regulating β-catenin, GSK3β is also known to phosphorylate and negatively regulate several transcription factors, one of which is Snail (131). In the presence of canonical Wnts, activated Disheveled prevents GSK3-dependent phosphorylation of its targets (96). Other mechanisms that also negatively regulate GSK3β include GSK3β phosphorylation on serine 9 by Akt, LiCl treatment, and 6-bromoindirubin-3'-oxime (BIO) treatment (14, 104, 114).

In this study I show that GSK3 regulates cadherin-11 expression in two ways. The first mechanism is through β-catenin independent regulation of cadherin-11 mRNA stability or sequestration. The second mechanism involves a β-catenin-dependent effect on cadherin-11 protein synthesis or stability.
II.C. MATERIALS AND METHODS

II.C.I. MATERIALS AND REAGENTS

LiCl (L9650), NaCl (S3014), and actinomycin D (A1410) were obtained from Sigma-Aldrich (Germany), meBIO (361556) and BIO (361550) from Calbiochem (San Diego, CA), anti-cadherin-11 (5B2H5) from Invitrogen (32-1700, Carlsbad, CA), anti-β-catenin antibody from BD Biosciences (610154, San Jose, CA), Dicer antibody from Abcam (ab14601, Cambridge, MA), anti-GSK3β antibody from Cell Signaling Technologies (9315, Boston, MA), anti-FLAG (M2) was from Sigma (F3165, Germany), anti-GAPDH was from Research Diagnostics Inc (TRK5G4-6C5, Flanders, NJ), anti-Snail (H-130) (sc-28199) antibody from Santa Cruz. The anti-eIF6 antibody S13 was a generous gift from Dr. Bifò and generated against a C-terminus peptide of eIF6 (4, 51). Small interfering RNA (siRNA) reagent (SMART pool) for human CTNNB1 (M-003482-00), Dicer (M-003483-00), and GSK3β (M-003010-03), were purchased from Dharmacon (Lafayette, CO). Non-specific (Scramble) siRNA was generated using forward: (5’-AAGCTCCTATAGCGTGATGGCCTGCCTGTC-3’) and reverse: (5’-CACCATAACGCTATAGGGAGCTTCCTGTC-3’) primers and the Silencer siRNA Construction kit (AM1620, Ambion, Austin, TX).

II.C.II. EXPRESSION VECTORS
Plasmid DNA encoding wild type (WT) β-catenin was used as previously described and DNA encoding wild type Snail was a kind gift from Dr. Mien-Chie Hung (88, 132). Dominant negative (DN) Snail was generated by PCR from the wild type Snail construct using primers described in Yamasaki et al. forward: (5’-CGGGATCCACTATGGCCTTCAACTGCAAATACTG-3’) and reverse: (5’-CGCTCGAGGCGGGACATCCTGAGCA-3’) and cloned into pCMV-Tag2 (Stratagene, La Jolla, CA) using *BamHI* and *XhoI* restriction sites (128). Cadherin-11 3’-UTR fragment was cloned from genomic DNA obtained from MDA-MB-231 cells using Expand High Fidelity PCR System (11732641001, Roche, Indianapolis, IN) according to the manufacturer’s protocol. For amplification of CDH11 3’-UTR Short forward: (5’-TGCTAGCTAAGTAAGTAACAATAACGATACAAATTT-3’) and reverse: (5’-CCGGATCCACGCGTGAATCTTGTCTGAAAAAACATTTG-3’) primers were used. For amplification of the CDH11 3’-UTR Long forward: (5’-TGCTAGCTAAGTAAGTAACAATAACGATACAAATTT-3’) and reverse: (5’-TGAGAATGTGTAATCCTTCACTGAG-3’) were used. These PCR products were ligated in place of the SV40 3’-UTR of pGL3-Promoter (Promega, Madison, WI).

II.C.III. CELL CULTURE AND TRANSFECTION

MDA-MB-231, Hs578T, BT549 breast cancer cells, PC-3 prostate cancer cells, and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) in 5 % CO₂ incubator at 37 °C. All
transient transfections of plasmid DNA and siRNA in MDA-MB-231 and PC-3 cells were performed with Amaxa electroporation system (Amaxa, Inc, Geithersburg, MD) according to the manufacturer’s protocol. Transient transfections for immunofluorescence were performed using Lipofectamine 2000 Transfection Reagent (11668-019, Invitrogen, Carlsbad, CA) and luciferase analysis using Lipofectamine 2000 (Invitrogen) for PC-3 cells, ProFection Mammalian Transfection System—Calcium Phosphate (E1200, Promega, Madison, WI) for MDA-MB-231 cells, and FuGENE 6 Transfection Reagent (11814443001, Roche, Switzerland) for HEK293 cells.

II.C.IV. RNA ISOLATION

II.C.IV.A. mRNA ISOLATION

MDA-MB-231 or PC-3 cells were incubated in the presence or absence of LiCl (20mM) or BIO (1 μM) for the time indicated. RNA was isolated using Trizol (15596-018, Invitrogen) combined with RNAeasy (74106, Qiagen, Valencia, CA) according to the manufacturer's instructions.

II.C.IV.B. TOTAL RNA ISOLATION (MICRORNA)

MDA-MB-231 cells incubated in the presence or absence of 20 mM LiCl for 24 hours. Total RNA was isolated using the miRVana RNA isolation kit (AM1562, Ambion).
II.C.V. REAL TIME QUANTITATIVE PCR

II.C.V.A. mRNA

Relative quantitation was used to evaluate the raw data obtained from real-time PCR (7900 HT real time PCR system, Applied Biosystems, Foster City, CA). Single-stranded cDNA was prepared using TaqMan Reverse Transcription Reagents (N808-0234, Applied Biosystems) following the manufacturer’s protocol. TaqMan Universal PCR Master Mix (4304437, Applied Biosystems) was used for all reactions. All primer/probe mixes (CDH11 Hs00156438_m1, GAPDH Hs99999905_m1) were obtained from Applied Biosystems and performed in triplicate. The samples were analyzed using the delta-delta Ct method of analysis (21). The final value obtained was a measure of the fold change in gene expression for the particular gene of interest between the treated sample and the untreated sample. Experiments were run in triplicate, and for all analyses a p-value of <0.05 was considered to be statistically significant.

II.C.V.B. MICRORNA

cDNA was generated by TaqMan MicroRNA Reverse Transcription kit (4366596, Applied Bioscience) following manufacturer’s protocol. Primer/probe mixes specific for each microRNA (RNU6B, 4373381; hsa-miR-19a, 4373099; hsa-miR-27a, 4373287; hsa-miR-33, 4373048; hsa-miR-101, 4373159; hsa-miR-133b, 4373172; hsa-miR-337, 4373044; hsa-miR-424, 4373201) were obtained from Applied Biosystems (Foster City, CA). TaqMan Universal Master PCR Mix (4304437, Applied Biosystems)
was used for all reactions. All experiments were performed in triplicate. The average value of the triplicate readings for each unknown was normalized to the corresponding value for U6 RNA. The samples were analyzed using the delta-delta Ct method of analysis (21). The final value obtained was a measure of the fold change in gene expression for the particular gene of interest between the treated sample and the untreated sample. For all analyses a P-value of <0.05 was considered to be statistically significant.

II.C.VI. RT-PCR

The exact length of the cadherin-11 3’UTR was determined with conventional RT-PCR. Total RNA was isolated with TRIZOL reagent (15596-018, Invitrogen). First strand cDNA was prepared by Superscript III First-Strand Synthesis System for RT-PCR (18080-051, Invitrogen) following manufacturer’s protocol. Expand High Fidelity PCR System (11732641001, Roche, Indianapolis, IN) was used according to the manufacturer’s protocol. For amplification of CDH11 2954, forward (5’-CTGTGTCTGGCGTTCTCAAG-3’) and reverse (5’-ACCTGACATACAGAGCCATGA-3’). For amplification of CDH11 2454, forward (5’-CTGTGTCTGGCGTTCTCAAG-3’) and reverse (5’-GCTGCATAGATGGTATAAAACAGTG-3’). For amplification of CDH11 1952, forward: (5’-CTGTGTCTGGCGTTCTCAAG-3’) and reverse: (5’-TGAGAATGTGTAATCCTTCACTGAG-3’) were used. For amplification of CDH11
1451, (5’-CTGTGTCTGGCGTTCTCAAG-3’) and reverse (5’-GGCCCAAGCATATTCTAAA-3’). For amplification of CDH11 931, forward (5’-TTGGCCTTAAGAAGCTGTGCTG-3’) and reverse (5’-AGCAGCAGACAGACAAACA-3’). For amplification of CDH11 420, forward (5’-TTGGCCTTAAGAAGCTGTGCTG-3’) and reverse (5’-TGCACTACATATAGGAGTCCAGAGTT-3’). For amplification of CDH11 1006, forward (5’-TGCTAGCTAAGTAAAGAACTGTGCTG-3’) and reverse (5’-CCGGATCCACGCCTGAATCTTGTCTGAAAAACATTTG-3’).

II.C.VII. CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS

One 15 cm tissue culture dish was plated with 85% confluent MDA-MB-231 cells for each condition. Cells were treated with 5 µg/ml actinomycin D for 30 minutes in serum-free DMEM. As specified, cells were incubated with meBIO or BIO for an additional 24 hours. 37% formaldehyde solution was added to each plate for a final concentration of 1.5% and incubated at 37ºC for 15 minutes. Plates were washed one time with PBS containing 0.125 M glycine and Complete Mini Protease Inhibitor Cocktail (11836153001, Roche), and then a second time with PBS plus inhibitor. Cells were collected and spun for at 2000 rpm, 4 ºC for 5 minutes. The pellet was resuspended in 1 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitors. Cells were then sonicated using a 15 second on, 45 second off program 4 times consecutively; then diluted 1:10 in ChIP Dilution Buffer
(0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH .1, 167 mM NaCl) plus protease inhibitor. The samples were then pre-cleared overnight at 4°C with 75 µl Protein A/G Plus-agarose beads (sc-2003, Santa Cruz) supplemented with 3 µl 10 mg/ml Sonicated Salmon Sperm DNA (201190, Stratagene, La Jolla, CA) and 13 µl 1 mg/ml BSA. Samples were then incubated with 10 µg RNA Polymerase II (N-20) (sc-899, Santa Cruz) overnight, tumbling at 4°C. Add 40 µl Protein A/G Plus-agarose beads for 2 hours rotating at 4°C. Samples were spun at 1000 rpm for 1 minute, then washed once with each: Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer. Samples were eluted twice in 100 µl in elution buffer (1% SDS, 0.1 M NaHCO₃) vortexing for 15 minutes at room temperature. 12 µl 5 M NaCl was added to eluate and incubated at 65°C overnight. Then 4 µl 0.5 M EDTA, 8 µl 1M Tris-HCl, pH 6.5 and 2 µl of 10 mg/ml proteinase K was added and incubated at 45°C for 1 hour. The samples were cleaned up using the QIAGEN PCR Clean up kit (QIAGEN). PCR was performed using TaKaRa Premix Ex Taq kit (RR039, TaKaRa, Otsu, Shiga, Japan). The final reaction contains: 1X Premix Ex Taq, 2.5 µM of each primer, 8% DMSO, and 20% processed DNA (or 20% of a 1:10 dilution of input). For amplification of
GAPDH, forward: (5’-TACTAGCGGTTCACGAGGC-3’) reverse: (5’-TCGAACAGGAGGCAGACAGGC-3’). For amplification of CDH11, forward: (5’-AAAGCAAAGGGAGGGGA-3’) reverse: (5’-AGGTACAAACCCCTCTGCT-3’) primers were used.

II.C.VIII. IMMUNOSTAINING

MDA-MB-231 or Hs578T cells were seeded on glass cover slips in growth medium and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Then cells were fixed with cold methanol at –20°C for 20 min for cadherin-11 staining. After blocking with 5 % BSA/PBS for 1 h at room temperature, fixed cells were incubated overnight at 4°C with anti-cadherin-11 antibody diluted 1:1000 (5B2H5) in 2% goat serum (GS)/PBS followed by incubation with Alexa Fluor 568-labeled anti-mouse IgG (1:1000) for 1 hour at room temperature. Cells were then incubated with 1:1000 Alexa Fluor 488-labeled mouse anti-β-catenin in 2% GS/PBS. Coverslips were mounted on glass slides using Vectashield with DAPI (H-1200, Vector Laboratories, Burlingame, CA). Nikon E600 Fluorescence Microscope with Hamamatsu Orca-100 and 20x, 40x, 60x objective lens and MetaMorph (version 6.1.5) imaging analysis software (Universal Imaging Corp.) were used to detect fluorescence.

II.C.IX. IMMUNOBLOTTING
Cells were treated for the indicated times. Cells were rinsed once with PBS and lysed with sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 7.5) containing 1 mM sodium orthovanadate, 0.05 M sodium fluoride, and Complete mini protease inhibitors (11836153001, Roche Applied Science, Indianapolis, IN). Cell lysates were boiled for 10 minutes. Protein concentration was determined with a Bio-Rad DC Protein Assay (500-0116, Bio-Rad, Hercules, CA). After SDS-poly acrylamide gel electrophoresis, proteins were transferred to Protran BA 83 Nitrocellulose (10402495, Germany). Membranes were blocked with % milk in Tris-Buffered Saline containing 0.1% Tween-20, and incubated with primary antibody overnight at 4ºC and subsequently with HRP-labeled secondary antibody. Proteins were visualized with ECL chemiluminescent reagents (RPN2106, Amersham Biosciences, Piscataway, NJ) or SuperSignal West Femto (34095, Pierce biotechnology Inc., Rockford, IL) using X-ray films (Denville Scientific Inc., Metuchen, NJ).

II.C.X. LUCIFERASE ASSAY ANALYSIS

Cells were plated at 2x10^5 in a 12-well plate in growth medium. Cells were transfected in triplicate with 1.8 ug of luciferase plasmid DNA and 0.2 ug of pCMV-Renilla (Promega). For cells treated with anti-Dicer siRNA, 100 nM siRNA was transfected together with Luciferase and Renilla using Amaxa nucleofection and collected and analyzed 48 hours later. Cells at varying densities were transfected using Amaxa nuclefection and 1.67 x10^5 cells (low) or 6.25x10^5 cells (high) were plated in triplicate.
in a 12-well plate. Cells treated with meBIO or BIO (1 µM) were transfected in triplicate using Fugene6 (Roche). 24 hours after transfection, cells were treated with either BIO or eBIO. 48 hours after treatment cells were collected. In all cases, cells were lysed using passive lysis buffer provided with the Dual-Luciferase Reporter Assay System (Promega). 20 µl of each sample was loaded in duplicate in a 96-well plate and analyzed using the Dual-Luciferase Reporter Assay System reagents (E1960, Promega). The plate was read on a Berthold MicroLumat Plus LB 96V (Germany) using WinGlow software (Berthold).
II.D. Results

II.D.I. Cell Density and Inhibition of GSK3 Regulate Cadherin-11 RNA and Protein Expression

MDA-MB-231 cells are a mesenchymal-like breast cancer cell line that expresses high levels of cadherin-11 and undetectable levels of other cadherins. In pilot experiments I noticed that cadherin-11 expression varied noticeably depending on the number of cells plated. Figure 7 shows that cells grown at higher cell densities had markedly increased cadherin-11 RNA (Figure 7A) and protein (Figure 7B) expression on a per cell basis. In *Xenopus*, cadherin-11 is regulated by xWnt8, although it is not known if the changes in cadherin-11 expression are direct or indirect or if either the canonical or non-canonical pathways are involved (40). To test if the canonical Wnt pathway can regulate cadherin-11 in MDA-MB-231 cells, we treated cells with lithium chloride (LiCl). LiCl treatment repressed cadherin-11 mRNA and protein levels and blocked the effects of increased cell density (Figure 7A, 7B). Cadherin-11 protein levels were also inhibited by LiCl in three other cell lines (Figure 7C). Remarkably, N-cadherin, which is also expressed by these cells, was unaffected by LiCl, strongly indicating that inhibition of GSK3 specifically regulated cadherin-11 expression. If the effects of LiCl are due to transient inhibition of GSK3 we anticipated that washout of LiCl would reverse its effects on cadherin-11. Figure 7D shows that this is indeed the case as cadherin-11 levels returned to control values 12 hours following LiCl washout. To confirm that the effects of LiCl were related to inhibition of GSK3, we treated cells BIO, a small molecule kinase inhibitor selective for GSK3α/β (Figure 7E, F). Inhibition
of GSK3 with BIO stabilized β-catenin, and like LiCl, repressed cadherin-11 mRNA and protein expression. Furthermore, siRNA directed against GSK3β decreased cadherin-11 expression in the absence of LiCl in cells with normally functioning GSK3, MDA-MB-231 cells (Figure 7G). Cells with low GSK3 activity, PC-3 cells, had no change in cadherin-11 expression in response to GSK3β knock down.

II.D.II. RECRUITMENT OF RNA POLYMERASE II TO THE CADHERIN-11 GENE IS UNAFFECTED BY LiCl OR BIO TREATMENT

Inhibition of GSK3 affects cadherin-11 expression at both the mRNA and protein level suggesting that the most likely mode of regulation is at the level of transcriptional repression. To test if GSK3 regulates cadherin-11 expression at the level of transcription, MDA-MB-231 cells were treated with the RNA polymerase II inhibitor actinomycin D and were compared to the results of cells treated with LiCl. Figure 8A shows that cadherin-11 mRNA levels declined following either LiCl or actinomycin D treatment. However, in this experiment, and on other occasions, we noticed that LiCl treatment repressed cadherin-11 mRNA levels more rapidly than actinomycin D suggesting an additional non-transcriptional level of regulation. If the effects of GSK3 inhibition on cadherin-11 mRNA have a transcriptional component, we would anticipate that recruitment of RNA polymerase II to the cadherin-11 gene to decrease. To test this hypothesis, we carried out RNA polymerase II chromatin immunoprecipitation assays (Pol II ChIP) using cadherin-11 specific primers which amplify a portion of intron 1 (Figure 8B). Figure 8C shows that, as expected,
actinomycin D markedly reduced the amount of Pol II recruited to both GAPDH and the cadherin-11 gene. However, even though cadherin-11 mRNA levels are markedly reduced by treatment of cells with BIO (Figure 7) it did not affect Pol II recruitment to the cadherin-11 gene (Figure 8C). In other experiments I found that members of the Snail/Slug family, transcriptional repressors known to be regulated by GSK3 and by β-catenin are not involved in cadherin-11 repression (Figure 9) (132). Taken together these experiments indicate that inhibition of GSK3 by LiCl or BIO influences cadherin-11 mRNA and protein levels largely independently of transcriptional repression.

II.D.III. OVEREXPRESSION AND KNOCKDOWN EXPERIMENTS DEMONSTRATE A ROLE FOR β-CATENIN IN MEDIATING THE EFFECTS OF GSK3β INHIBITION ON CADHERIN-11 PROTEIN BUT NOT mRNA

Perhaps the best known substrate of GSK3 is β-catenin and many studies have shown that Wnt-mediated inhibition of GSK3 stabilizes and activates cytoplasmic β-catenin (42, 100). Indeed this is the basis of the canonical Wnt pathway in which activated β-catenin co-activates TCF target genes such as cyclin D1 and c-myc (41, 119). Although the effects of inhibition of GSK3 on cadherin-11 are not mediated transcriptionally it is possible that stabilization of β-catenin may repress cadherin-11 mRNA and/or protein levels using an alternative mechanism. To test this we over-expressed and knocked down β-catenin in MDA-MB-231 breast cancer cells and PC-3 prostate cancer cells and measured cadherin-11 mRNA levels. Untreated MDA-MB-231 cells have very low levels of activated β-catenin protein and signaling activity whereas PC-3 cells have high levels of nuclear activated β-catenin as a result of a PTEN
mutation (78). Quantitative real-time PCR (QPCR) analyses showed that cadherin-11 mRNA levels and their repression by BIO were unaffected by either over-expression or knockdown of β-catenin in MDA-MB-231 and PC-3 cells (Figure 10A and 10B, respectively). In contrast, knockdown of β-catenin in PC-3 cells, which express endogenous activated β-catenin, increased cadherin-11 protein levels (Figure 10C). β-catenin knockdown was unable to reverse the suppressive effect of BIO in MDA-MB-231 cells, cells with low endogenous levels of activated β-catenin (Figure 10C). If removal of β-catenin attenuates the effects of GSK3 inhibition on cadherin-11 protein we reasoned that over-expression of β-catenin might repress cadherin-11 protein in the absence of BIO. Figure 10D demonstrates that exogenous expression of β-catenin in MDA-Mb-231 cells significantly repressed cadherin-11 protein levels detected by Western blot (Figure 10D) and immunocytochemistry (Figure 10E). Taken together these data suggest that inhibition of GSK3 differentially represses cadherin-11 mRNA and protein and that activation of β-catenin directly influences cadherin-11 protein but not mRNA levels. We recently showed that β-catenin itself was regulated at the protein but not RNA level by the ribosome binding protein eIF6 (51). However, over-expression and knockdown of eIF6 did not influence the ability of BIO to repress cadherin-11 mRNA or protein (Figure 11).

II.D.III. THE CADHERIN-11 3’UNTRANSLATED REGION (UTR) IS HIGHLY CONSERVED AND REGULATED BY DICER
In most situations regulation of mRNA stability and its capacity for translation are mediated by the 3’-untranslated region (UTR). If the cadherin-11 3’-UTR is important in the normal regulation of cadherin-11 expression we might expect it to be conserved across species. Table 1 shows the percent identity of the human cadherin-11 and E-cadherin transcripts and 3’-UTRs compared with other species. Remarkably for a region that is not translated the cadherin-11 3’-UTR is almost completely conserved across species. Figure 12A shows the sequence of the human cadherin-11 3’-UTR, with the red highlighted sequences denoting the cloned sequence. Additionally, the bolded sequences denote the poly-A signal and site, respectively. Furthermore, regulatory sequences are denoted by blue highlighted sequences indicating Shaw-Kamens sequences, also known as AU-rich elements (AREs), and underlined sequences denoting putative microRNA binding sites within this region (109). Additionally, analysis of the secondary structure of this region revealed a high degree of stem loop structures more akin to tRNA than mRNA (Figure 12B).

These observations strongly suggest that the cadherin-11 3’-UTR has a conserved regulatory function. To investigate the function of the cadherin-11 3’-UTR, it was cloned from MDA-MB-231 genomic DNA. A series of stop codons were included at the 5’-end of the construct followed by the DNA sequence directly 3’ to the stop codon, the 3’-UTR. This region includes the poly-A signal, the poly-A site, and terminates with additional non-coding sequence at the 3’-end of the gene. The sequence denoted by NCBI as the 3’-UTR of cadherin-11 was cloned using the red highlighted
primer sequences (Figure 12A). This PCR product was cloned into the pGL3-Promoter reporter vector and named luc-CDH11 3’-UTR Short (Figure 12C). Figures 12D, 12E and 12F show the activity of this reporter under various conditions. To investigate if microRNA influenced the activity of luc-CDH11 3’-UTR Short we knocked down Dicer, which is essential for the production of microRNAs. Removal of Dicer significantly increased the activity of luc-CDH11 3’-UTR Short indicating that microRNAs regulate the exogenous 3’UTR, but the endogenous 3’UTR is not significantly regulated by microRNAs (Figure 12D). However, neither cell density (Figure 12E) nor inhibition of GSK3 (Figure 12F) affected the activity of this reporter. Consistent with a role for miRNA, several putative miRNA binding sites exist in this region as denoted by the underlined sequences in Figure 12A. However, none of these predicted miRNAs was regulated by inhibition of GSK3 (Figure 13). These data demonstrate that although miRNAs can regulate luc-CDH11 3’-UTR Short this is not the mechanism whereby density or GSK3 inhibition regulate cadherin mRNA levels. Unlike siRNAs, which always direct mRNA degradation, certain miRNAs regulate protein translation without affecting mRNA levels (19, 20, 91). To test this I examined the effects of Dicer knockdown on cadherin-11 protein and mRNA levels. Figure 14 shows that even in the face of Dicer knockdown, endogenous cadherin-11 mRNA and protein levels were unchanged and the repressive effects of LiCl were not reversed.

II.D.IV. ADDITIONAL 3’UTR ELEMENTS EXIST AND REGULATE CADHERIN-11:
The preceding experiments were carried out with a region of the cadherin 3’UTR identified from the NCBI database and includes approximately 1 kb of the 3’UTR of cadherin-11 including a traditional poly-A signal and site. A search of the Ensembl database uncovers a sequence identified as the cadherin-11 3’UTR of roughly 3 kb which includes a second poly-A signal 2363 bp from the stop codon (Figure 15A). The traditional poly-A signal sequences (AAUAAA) within the 3’UTR are bolded in Figure 15A. To determine the approximate length of the cadherin-11 3’UTR in cells actively expressing cadherin-11, RT-PCR was used. RNA was isolated from PC3 metastatic prostate cancer cells (Figure 15B), MDA-MB-231 cells (data not shown), and MRC5 lung fibroblast cells (data not shown) for analysis. Primers were designed approximately every 500 bp of the Ensembl sequence, as noted by the highlighted sequences in Figure 15A, to make progressively shorter PCR products. Analysis reveals that in all three cell lines the primers designed to produce a PCR product of 1451 bp are able to amplify, but the primers designed to make a product size of 1952 bp are unable to amplify (Figure 15B). Although, this region does not include a poly-A signal or addition site within a reasonable distance from its end, one does exist at position 2363 indicating that this in fact may be an alternate 3’-UTR. Despite not containing a traditional poly-A signal, the longer cadherin-11 3’-UTR was cloned into pGL3-Promoter using first red primer and second green primer depicted in Figure 15A and named luc-CDH11 3’-UTR Long. This construct was transfected into HEK293 cells (Figure 16A), MDA-MB-231 cells (Figure 16B), and PC-3 cells (Figure 16C), and
in each case, cells transfected with luc-CDH11 3'-UTR Long had statistically significant less luciferase activity than cells transfected with either pGL3-Promoter (control) or luc-CDH11 3'-UTR Short. The luciferase activity of pGL3-Promoter compared to luc-CDH11 3'-UTR Short is significantly different in cells expressing cadherin-11 (MDA-MB-231 and PC-3) but not in non-cadherin-11 expressing cells (HEK293). This implies that luc-CDH11 3'-UTR Short is regulated by a mechanism that is inactive in non-cadherin-11 expressing cells. The marked reduction in activity with the addition of approximately 1000 bp of supposed cadherin-11 3’-UTR led me to suspect that this may not be an actual cadherin-11 3’UTR. Unfortunately, RT reactions often produce products that are truncated at the 3’ end, therefore I think that the actual poly-A addition signal, site, and official terminus of the cadherin-11 transcript is further down-stream of the 1952 primer.

II.E. DISCUSSION

The majority of patients dying from breast or prostate cancer have metastases to the skeleton. Although bone metastases are incurable, patients survive several years suffering serious morbidity, including fractures, spinal cord compression, severe bone pain, and hypercalcemia (11, 37). In the adult organism, cadherin-11 is strongly expressed in bone as well as certain metastatic cancers, particularly those inclined to metastasize to bone (7, 37, 39). Once in the bone, it is possible that these cadherin-11-expressing tumor cells activate either osteoclasts or osteoblasts, depending on the type
of cancer metastasis, leading to bone remodeling (11, 37, 99). As the bone is remodeled, growth factors are released into the matrix further stimulating the tumor cells by creating a fertile environment for growth (38, 39). These changes are reminiscent of those that occur during rheumatoid arthritis development, a disease that is also characterized by an important role for cadherin-11 in the activated synoviocyte (57, 63). Given its role in these important diseases, I sought to determine how cadherin-11 expression is regulated. In the present study I show that cadherin-11 mRNA and protein levels are increased by high cell density and are markedly regulated by the activity of the ubiquitous serine/threonine kinase GSK3.

II.E.I. CADHERIN-11 REGULATION BY GSK3

GSK3 is known to regulate many pathways, and its activity is crucial to cellular function. In this instance, GSK3 activity plays an important role in the regulation of adhesion via cadherin-11. GSK3 was first identified as the enzyme that phosphorylates glycogen synthase and was later found to be involved in the insulin and Wnt pathways (26). Since that time, GSK3 has been shown to interact with an overwhelmingly large number of substrates (at least 50 are known), and therefore its activity must be intricately regulated.

Four distinct mechanisms of GSK3 regulation are known. They are: phosphorylation of GSK3 itself, phosphorylation of its substrates, protein complexes association, and its subcellular localization. The best studied mechanism by which
GSK3 is regulated by Akt-dependent serine phosphorylation. Growth factors, such as insulin, activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. PI3K converts PIP$_2$ to PIP$_3$, a critical step necessary for the activation of PKB/Akt. PTEN is a phosphatase commonly mutated in cancers that is necessary for the dephosphorylation of PIP$_3$ thereby inhibiting Akt activation. Once activated, Akt acts on many cellular proteins including mTOR, caspase 9, Bad, IKK, and GSK3. Akt phosphorylates GSK3$\beta$ on serine 9 and GSK3$\alpha$ on serine 21 to inhibit its kinase activity. Understanding GSK3 regulation by the Akt pathway is important to the present study, because the PTEN mutation in PC-3 prostate cancer cells results in activated Akt, GSK3 inhibition, and activation of endogenous $\beta$-catenin.

GSK3 activity is important in the regulation of cell migration and adhesion. For example, GSK3 helps to control microtubule networks, and its inactivation leads to epithelial cell migration. GSK3 also phosphorylates focal adhesion kinase (FAK) reducing its activity and inhibiting cell migration. $\beta$-catenin, a well-studied GSK3 target, transcriptionally regulates many proteins involved in cell cycle progression and cell survival. GSK3 inhibition leads to an accumulation of $\beta$-catenin and a rapid increase in $\beta$-catenin target genes. Importantly, GSK3 is central to the promotion of inflammation in various inflammatory diseases including colitis and arthritis.

The second well-defined mechanism of GSK3 regulation is the canonical Wnt pathway. In this case GSK3 is bound to a protein complex regulating its activity. In the absence of the Axin-APC-GSK3 complex, GSK3 is no longer able to bind and
phosphorylate \( \beta \)-catenin. In this study I show for the first time that inhibition of GSK3 and stabilization of \( \beta \)-catenin results in marked changes in cadherin-11 expression. Remarkably, these effects are independent of transcription.

**II.E.II. A ROLE FOR GSK3 AND \( \beta \)-CATENIN IN mRNA AND PROTEIN HOMEOSTASIS**

Although many studies have observed that Wnt-GSK3-mediated activation of \( \beta \)-catenin results in accumulation of nuclear and cytoplasmic \( \beta \)-catenin, an overwhelming number of studies concentrate on the function of \( \beta \)-catenin as a transcriptional co-activator of TCF, and more recently nuclear receptors, in the nucleus (22, 23, 82, 108, 111). However, \( \beta \)-catenin has significant homology to the RNA binding protein pumilio and several recent studies have pointed to an important role for \( \beta \)-catenin in post-transcriptional regulation (64-66, 89). For example, \( \beta \)-catenin regulates VEGF-D, cyclooxygenase-2 and cyclin D1 mRNA stability potentially by interaction with their respective 3’-UTRs (64-66, 89). The ribosome-associated factor eIF6 associates with \( \beta \)-catenin and the complex may have a role in translational regulation (10, 51). In addition, \( \beta \)-catenin can regulate alternative splicing of estrogen receptor \( \beta \) (ER\( \beta \)) in colon cancer cells (64). Although our data show that the known cadherin-11 3’-UTR is not subject to regulation by GSK3 or \( \beta \)-catenin, it is likely that additional regulatory elements exist in this region of the gene [http://www.ensembl.org/Homo_sapiens/exonview?db=vega;transcript=OTTHUMT00000268755](http://www.ensembl.org/Homo_sapiens/exonview?db=vega;transcript=OTTHUMT00000268755). Finally, even though the endogenous activity of the cadherin-11 3’-UTR is
regulated by Dicer, cadherin-11 mRNA and protein regulation by GSK3 is not, ruling out a role for miRNAs in this process. Taken together these data point to an important role for GSK3 and β-catenin in cadherin-11 mRNA and protein homeostasis and may lead to the development of therapeutics for diseases such as, metastatic prostate and breast cancer and rheumatoid arthritis, that are characterized by elevated cadherin-11. Indeed inhibition of GSK3 or ablation of the GSK3β gene ameliorates inflammation dependent arthritis (43).

II.F. ACKNOWLEDGEMENTS

This work was supported by DOD Idea Award DAMD17-01-1-0245 (SWB) and DOD Prostate Cancer Research Predoctoral Traineeship Award W81XWH-05-1-0559 (AKF).

II.G. FIGURE LEGENDS

FIGURE 7: DENSITY INCREASES CADHERIN-11 AND GSK3β INHIBITORS DECREASE CADHERIN-11 EXPRESSION. A and B: MDA-MB-231 cells were plated at three densities, 50%, 80%, and 90% confluency, called low, medium, and high, respectively. The cells were allowed to grow overnight in serum-free medium. 24 hours after plating cells were treated with 20 mM NaCl (N) or 20 mM LiCl (L). 24 hours after treatment, RNA and protein were collected for real-time PCR (A) and Western (B) analysis. C: Cancer cells BT549, Hs578T, PC-3, and Kato-III were plated at a medium density and allowed...
to adhere. Cells were then treated with 20 mM LiCl or NaCl control. 48 hours after transfection protein was collected for Western blot analysis. D: MDA-MB-231 cells were plated at a medium density, serum starved overnight, and treated with 20 mM NaCl or 20 mM LiCl. 24 hours after treatment, the cells were washed once with PBS and maintained in serum-free medium containing only 20 mM NaCl. RNA was collected at the times indicated for real-time PCR analysis. E and F: MDA-MB-231 cells were plated at a medium density and serum starved overnight. 16 hours after plating, the cells were treated with 1 μM meBIO (control), 1 μM BIO, or 20 mM LiCl. RNA (E) or protein (F) was collected at the designated times and analyzed using real-time PCR (E) and Western blot analysis (F). G: MDA-MB-231 and PC-3 cells were transfected with either non-specific scrambled siRNA (siScramble), or siRNA directed against GSK3β (si GSK3). 48 hours after transfection protein was isolated for Western blot analysis.

**Figure 8: Transcriptional Mechanism of Cadherin-11 Regulation.** A: MDA-MB-231 cells were grown at a medium density for 16 hours in the absence of serum. The cells were then pretreated with 5 μg/ml actinomycin D or an equivalent volume of ethanol (untreated). 30 minutes later the cells were treated with either 20 mM NaCl (control) or LiCl. At the indicated time points, RNA and protein were collect for real-time PCR analysis. B: Diagram of cadherin-11 genomic structure. Arrows indicate the approximate location of primers used for ChIP analysis. C: MDA-MB-231 cells were
treated with 1 µM meBIO or BIO for 24 hours. Genomic DNA was then harvested for RNA polymerase II ChIP analysis followed by PCR specific to GAPDH and cadherin-11.

**Figure 9 (Supplementary Figure 1 in original manuscript): Snail fails to regulate cadherin-11 expression.**

A: Breast cancer cells, BT549, Hs578T, and MDA-MB-231 (MDA231), were plated at a medium density. Cells were transfected with pcDNA3, Flag-wt-Snail, or Flag-DN-Snail. 48 hours after transfection protein was collected for Western blot analysis. B: Hs578t breast cancer cells were transfected with either control vector (i-iii) or wild type Snail (iv-vi). 48 hours after transfection, the cells were immunostained for cadherin-11 (CDH11) or Snail.

**Figure 10: β-catenin as a regulator of cadherin-11 expression.**

A and B: MDA-MB-231 (A) and PC-3 (B) cells were transfected with either pcDNA3, wild type β-catenin (wt β-cat), non-specific scrambled siRNA (siScramble), or siRNA directed against CTNNB1 (si β-cat). 24 hours after transfection cells were treated with 1 µM BIO or meBIO (control). 48 hours after transfection RNA was isolated for real-time PCR analysis. C: MDA-MB-231 and PC-3 cells were transfected with either non-specific scrambled siRNA (siScramble) or siRNA directed against CTNNB1 (si β-cat). 24 hours after transfection cells were treated with 1 µM BIO or meBIO (control). 48 hours after transfection protein was isolated for Western blot analysis. D: MDA-MB-
231 cells were plated at a medium density and allowed to grow overnight in serum-containing medium. The cells were transfected with wild type β-catenin. Protein was collected 24 hours and 48 hours after transfection for Western blot analysis. E: MDA-MB-231 cells were transfected with wild type β-catenin. 48 hours after transfection, the cells were immunostained for cadherin-11 (CDH11) and β-catenin. (* indicates a p-value > 0.05)

**Figure 11 (Supplementary Figure 2 in original manuscript):** eIF6 fails to regulate cadherin-11 expression. A: MDA-MB-231 cells were transfected with non-specific siRNA (siScramble), siRNA directed against eIF6 (si eIF6), pcDNA3, or wt eIF6. 24 hours after transfection, cells were treated with 20mM NaCl or LiCl. 48 hours after transfection cells were harvested for real-time PCR analysis. B: MDA-MB-231 cells were transfected with non-specific siRNA (siScramble), or siRNA directed against eIF6 (si eIF6). Cells were collected for Western blot analysis at the indicated time points.

**Figure 12: In silico evaluation of the cadherin-11 3’-UTR.** A: Sequence of cadherin-11 3’-UTR according to the NCBI database (NM_001797). Bolded sequences indicate the poly-A signal and site respectively (as designated by NCBI). Blue highlighted sequences indicate Shaw-Kamens (ARE) destabilizing sequences. Underlined sequences indicate putative microRNA binding sites (e.g. hsa-mir-27a, hsa-
mir-337, hsa-mir-33, hsa-mir-19a/b, hsa-mir-101, hsa-mir-424, hsa-mir-133a/b, hsa-mir-339, hsa-mir-342). Red highlighted sequences indicate the primers used to construct the pGL3-CDH11-3’-UTR. B: Predicted secondary structure of the cadherin-11 3’-UTR (as predicted by GeneBee). C: Schematic of cadherin-11 3’-UTR cloned sequence. D: MDA-MB-231 and PC-3 cells were transfected with pGL3-CDH11 3’-UTR and pCMV-Renilla along with either non-specific siRNA (siScramble) or siRNA directed against Dicer (siDicer). 48 hours after transfection, cells were lysed using passive lysis buffer and luciferase activity was analyzed. E: MDA-MB-231 cells were transfected with pGL3-CDH11 3’-UTR and pCMV-Renilla and plated at 25% (Low) or 85% (high) confluency. 48 hours after transfection cell were lysed using passive lysis buffer and luciferase activity was analyzed. F: HEK 293 cells were transfected with pGL3-CDH11 3’-UTR and pCMV-Renilla. 24 hours after transfection, cells were treated with 1μM BIO or meBIO. 48 hours after transfection cells were were lysed using passive lysis buffer and luciferase activity was analyzed. Luciferase activity was normalized to renilla activity. (* indicates a p-value > 0.05).

**Figure 13 (Supplementary Figure 3 in original manuscript): MicroRNAs predicted to bind to the cadherin-11 3’-UTR.** A through F: MDA-MB-231 cells were treated with 20 mM LiCl or NaCl (control). 24 hours after treatment, total RNA was harvested for real-time PCR analysis.
**FIGURE 14: THE EFFECT OF DICER KNOCKDOWN ON CADHERIN-11 EXPRESSION.** A and B: MDA-MB-231 cells were transfected with non-specific siRNA (siScramble), or siRNA directed against Dicer (si Dicer). Cells were collected for Western (A) or real-time PCR analysis (B) at the indicated time points. C and D: MDA-MB-231 cells were transfected with non-specific siRNA (siScramble), or siRNA directed against Dicer (si Dicer). 48 hours after transfection, cells were treated with 10 ng/ml TGFβ1 or 20 mM LiCl or NaCl control. 72 hours after transfection cells were collected for Western (C) and real-time PCR analysis (D).

**FIGURE 15 (NOT INCLUDED IN ORIGINAL MANUSCRIPT): AN ALTERNATIVE 3’UTR FOR CADHERIN-11.** A: Sequence of cadherin-11 3’UTR according to the Ensembl database. Bolded sequences indicate the poly-A signals and site respectively. Blue highlighted sequences indicate Shaw-Kamens (ARE) destabilizing sequences. Green and red highlighted sequences indicate primer sequences used for RT-PCR. Underlined sequence indicates putative microRNA binding sites (e.g. hsa-mir-27a, hsa-mir-337, hsa-mir-33, hsa-mir-19a/b, hsa-mir-101, hsa-mir-424, hsa-mir-133a/b, hsa-mir-339, hsa-mir-342). Red highlighted sequence denotes the primer sets used to clone pGL3-CDH11-3’UTR reporter constructs. B: RT-PCR of PC3 RNA using primers specified in A.
Figure 16: The destabilizing effects of the cadherin-11 3’UTR. A, B, and C: Cells were plated at a medium density and allowed to adhere for 16 hours. HEK 293T cells (A), MDA-MB-231 cells (B), or PC3 (C) were transfected with empty vector (pGL3), luc-CDH11-3’UTR Long (Long), or luc-CDH11-3’UTR Short (Short). 48 hours after treatment cells were lysed using passive lysis buffer, and luciferase activity was analyzed. Luciferase activity was normalized to renilla activity. (* indicates a p-value > 0.05)
II.H. Figures

Figure 7. Density increases cadherin-11 and GSK3β inhibitors decrease cadherin-11 expression

A. CDH11 RNA [Fold Change] comparison between NaCl and LiCl treatments at different cell densities (Low, Medium, High).

B. Western blot showing Cadherin-11 and Actin expression levels under NaCl and LiCl treatments.

C. Western blot analysis of N-cadherin, CDH11, and GAPDH in BT549, Hs578t, PC-3, and Kato-III cell lines under NaCl and LiCl treatments.

D. Graph illustrating CDH11 RNA [Fold Induction] over time (Hours) for NaCl and LiCl treatments.

E. Graph showing CDH11 RNA [Fold Change] over time (Hours) for meBIO and BIO treatments.

F. Western blot analysis of CDH11, Total b-cat, and GAPDH with 1 uM meBIO and 1 uM BIO treatments.

G. Western blot analysis of CDH11, GSK3β, and GAPDH in MDA-MB-231 and PC-3 cells with Scramble and siGSK3β treatments.
FIGURE 8. TRANSCRIPTIONAL MECHANISM OF CADHERIN-11 REGULATION

A

B

C

172 bp

Input RNA Pol II Beads alone

meBIO BIO ActD ActD+BIO meBIO BIO ActD ActD+BIO meBIO BIO ActD ActD+BIO

GAPDH

CDH11
FIGURE 9 (SUPPLEMENTARY FIGURE 1 IN ORIGINAL MANUSCRIPT). SNAIL FAILS TO REGULATE CADHERIN-11 EXPRESSION

A

B
Figure 10. β-catenin as a regulator of Cadherin-11 expression

A

B

C

D

E

CDH11

β-catenin

GAPDH

MDA-MB-231

PC-3

Control

β-catenin

CDH11

Merged
FIGURE 11 (SUPPLEMENTARY FIGURE 2 IN ORIGINAL MANUSCRIPT). eIF6 FAILS TO REGULATE CADHERIN-11 EXPRESSION

A

![Bar chart showing CDH11 RNA fold change with and without eIF6 expression under NaCl and LiCl conditions.](image)

B

![Western blot showing CDH11 and eIF6 expression over time.](image)
### Table 1. Sequence Identity Evaluation for E-cadherin and Cadherin-11

<table>
<thead>
<tr>
<th>Species</th>
<th>E-cadherin coding sequence</th>
<th>E-cadherin 3'UTR</th>
<th>Cadherin-11 coding sequence</th>
<th>Cadherin-11 5'UTR</th>
<th>Cadherin-11 3'UTR</th>
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<td>89</td>
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<td>94</td>
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<td><em>R. norvegicus</em></td>
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<td>90</td>
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<td>94</td>
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<tr>
<td><em>P. troglodytes</em></td>
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<td>98</td>
<td>99</td>
<td>98</td>
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<td>64</td>
<td>N/A</td>
<td>80</td>
<td>59</td>
<td>86</td>
</tr>
</tbody>
</table>
FIGURE 12. *In silico* evaluation of the Cadherin-11 3'UTR

A

B

*Free Energy of Structure* = -115.9 kcal/mol
FIGURE 13 (SUPPLEMENTAL FIGURE 3 IN ORIGINAL MANUSCRIPT). MICRORNAs PREDICTED TO BIND TO THE CADHERIN-11 3'UTR

A

B

C

D

E

F

hso-mir-19 RNA [Fold Change]

hso-mir-27a RNA [Fold Change]

hso-mir-10 RNA [Fold Change]

hso-mir-33 RNA [Fold Change]

hso-mir-424 RNA [Fold Change]

hso-mir-133 RNA [Fold Change]
Figure 14. The effect of Dicer knockdown on cadherin-11 expression

A

B

C

D

CDH11 RNA [Fold Change]
Figure 15 (Not included in original manuscript). An alternative 3′UTR for Cadherin-11

A

CAATAACGATACAAATTTGGCTTAAGAACTGTGTCTGGCGTTCTCAAGAATCTAGAAGATGTGTAAACAGGTATTTTTTTAAATCAA
GGAAAGGCT

B

Genomic DNA

RT-PCR

66
FIGURE 16 (Not included in original manuscript) The destabilizing effects of the cadherin-11 3'UTR

A

![Graph A]

B

![Graph B]

C

![Graph C]
THE WORK PRESENTED IN CHAPTER III WILL BE PUBLISHED AS FOLLOWS:

FARINA AK, LECHLEIDER RJ, BYERS SW. TGFβ1 PATHWAY ACTIVATION ATTENUATES CADHERIN-11 EXPRESSION USING A SMAD-INDEPENDENT MECHANISM. IN PREPARATION.

III.A. ABSTRACT

The cell-cell adhesion molecule cadherin-11 is important in embryogenesis and bone morphogenesis, invasion of cancer cells, lymphangiogenesis, homing of cancer cells to bone, and rheumatoid arthritis. However, very little is known about the regulation of cadherin-11 expression. In this study we show that treatment of MDA-MB-231 breast cancer cells with TGFβ1 reduces basal levels of cadherin-11 mRNA and protein and attenuates the increase in cadherin-11 that occurs with increased cell density. In most cases TGFβ1 regulates gene transcription through Smad activation. However, RNA Polymerase II chromatin immunoprecipitation experiments showed that TGFβ1 does not affect transcription of the cadherin-11 gene in MDA-MB-231 cells. and knock-down of Smads 2, 3, 4 and 7 eliminated them as mediators of TGFβ1 regulation of cadherin-11. Treatment of cells with inhibitors of Smad-independent TGFβ1 pathways showed that inhibition of p38 MAPK or its knockdown mimicked the effect of TGFβ1 on cadherin-11 mRNA levels. Additionally, treatment of MDA-MB-231 cells with SB-431542, a TGFβ type I receptor (TβRI) inhibitor completely reverses the suppressive effect of TGFβ1 and increases endogenous cadherin-11 RNA. Taken together these data show that TGFβ1 binding to the TGFβ1 receptor and inactivation of p38 regulate cadherin-11 expression.
III.B. INTRODUCTION

TGFβ is known to play a dual role in cancer progression. Early in tumorigenesis, TGFβ inhibits growth and proliferation acting as a tumor suppressor. Prior to carcinogenesis TGFβ1 delays or prevents the development of cancer (59, 92). As tumorigenesis progresses, cells either lose responsiveness to, or overcome the growth-inhibitory effects of TGFβ. In late stage carcinogenesis, TGFβ1 often promotes motility and invasiveness. During TGFβ-mediated epithelial-to-mesenchymal transition (EMT), cell-cell adherens junctions decrease as E-cadherin is downregulated. Additionally, markers of motility such as stress fibers, focal adhesions and N-cadherin are upregulated (75).

Although TGFβ1 is expressed throughout the organism, its expression in bone and joints is of particular interest. Typically, TGFβ1 is released into the extracellular matrix as the bone is remodeled providing a constant source of cytokine (37). Further, TGFβ1 increases inflammation and has been associated with arthritis. Various members of the TGFβ1 signal transduction pathway, including TGFβ1 itself, are upregulated in synovial fibroblasts from patients with rheumatoid arthritis more so than fibroblasts from patients with osteoarthritis (5, 95).

Cadherin-11, an adhesion molecule involved in the formation of adherens junctions, is expressed in the organism during development and then only in select tissues in the adult organism. Specifically, cadherin-11 is expressed in osteoblasts in the bone and fibroblasts in the synovium (52, 53, 57, 63). In addition cadherin-11 is
also expressed by highly metastatic cancers which frequently metastasize to bone tissue (94). The link between cancers that metastatize to the bone and cadherin-11 has led us to investigate the role TGFβ1 plays in regulating cadherin-11 in cancerous cells.

The relationship between cadherin-11 and TGFβ1 has been poorly defined. Getsios et al. found that cadherin-11 RNA and protein expression increased in human cultured extravillous cytotrophoblasts cells in response to TGFβ1 (32). Trophoblast cells express a baseline level of cadherin-11, which is enhanced with TGFβ1 treatment. Therefore it is the goal of this report to shed light on the relationship between these two molecules.

III.C. MATERIALS AND METHODS

III.C.I. MATERIALS AND REAGENTS

TGFβ1 (100-21) was obtained from PeproTech (Rocky Hill, NJ), SB-431542 (S4317) and actinomycin D (A1410) were obtained from Sigma-Aldrich (Germany). SB-202190 (559388), SP-600125 (420119), PD-098059 (513000), and H-89 (371963) were obtained from Calbiochem (Gibbstown, NJ). Anti-cadherin-11 (5B2H5) from Invitrogen (32-1700, Carlsbad, CA), anti-GAPDH was from Research Diagnostics Inc (TRK5G4-6C5, Flanders, NJ), anti-α-tubulin (037K4827, Sigma-Aldrich), anti-Smad2 (sc-6200, Santa Cruz, Santa Cruz, CA), anti-Smad3 (sc-8332, Santa Cruz, Santa Cruz, CA), anti-Smad4 (MAB20971, R&D Systems, Minneapolis, MN), anti-Smad7 (sc-11392, Santa Cruz, Santa Cruz, CA), anti-Hsp 72/73 (HSP01, Calbiochem, Gibbstown,
NJ), anti-p38 MAPK (9212, Cell Signaling, Danvers, MA). Small interfering RNA (siRNA) reagent (SMART pool) for human Smad2 (M-003561-01), Smad3 (M-020067-00), Smad4 (M-003902-01), Smad7 (M-020068-01), and MAPK14 (M-003512-06) were purchased from Dharmacon (Lafayette, CO). Non-specific (Scramble) siRNA was generated using forward: (5'-AAGCTCCTATAGCGTATGGTGCCTGTCTC-3’) and reverse: (5’-CACCATACGCTATAGGAGCTTCCTGTCTC-3’) primers and the Silencer siRNA Construction kit (AM1620, Ambion, Austin, TX).

III.C.II. EXPRESSION VECTORS

Plasmid DNA encoding wild type (WT) Smad2 and Smad3 were a gift from Rik Derynck and used as previously described in (12). Wild type (WT) Smad4 was a gift from Dr. Robert Lechelider and Dr. Shiyou Chen and used as previously described in (15).

III.C.III. CELL CULTURE AND TRANSFECTION

MDA-MB-231 breast cancer cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in 5% CO2 incubator at 37 °C. All transient transfections of plasmid DNA and siRNA in MDA-MB-231 were performed with Amaxa electroporation system (Amaxa, Inc, Geithersburg, MD) according to the manufacturer’s protocol.
III.C.IV. RNA ISOLATION

MDA-MB-231 or PC-3 cells were incubated in the presence or absence of TGFβ1 for the indicated lengths of time. RNA was isolated using Trizol (15596-018, Invitrogen) combined with RNAeasy (74106, Qiagen, Valencia, CA) according to the manufacturer's instructions.

III.C.V. REAL TIME QUANTITATIVE PCR

Relative quantitation was used to evaluate the raw data obtained from real-time PCR (7900 HT real time PCR system, Applied Biosystems, Foster City, CA). Single-stranded cDNA was prepared using TaqMan Reverse Transcription Reagents (N808-0234, Applied Biosystems) following the manufacturer’s protocol. TaqMan Universal PCR Master Mix (4304437, Applied Biosystems) was used for all reactions. All primer/probe mixes (CDH11 Hs00156438_m1, GAPDH Hs99999905_m1, PAI-1 Hs01126606_m1, MAPK14 Hs00176247_m1) were obtained from Applied Biosystems and performed in triplicate. The samples were analyzed using the delta-delta Ct method of analysis (21). The final value obtained was a measure of the fold change in gene expression for the particular gene of interest between the treated sample and the untreated sample. Experiments were run in triplicate, and for all analyses a p-value of <0.05 was considered to be statistically significant.
III.C.VI. CHROMATIN IMMUNOPRECIPITATION (ChIP) ANALYSIS

One 15 cm tissue culture dish was plated with 95% confluent MDA-MB-231 cells for each condition. Cells were treated with 5 µg/ml actinomycin D for 30 minutes in serum-free DMEM. As specified, cells were incubated with 10 ng/ml TGFβ1 for an additional 24 hours. 37% formaldehyde solution was added to each plate for a final concentration of 1.5% and incubated at 37°C for 15 minutes. Plates were washed one time with PBS containing 0.125 M glycine and Complete Mini Protease Inhibitor Cocktail (11836153001, Roche), and then a second time with PBS plus inhibitor. Cells were collected and spun for at 2000 rpm, 4 °C for 5 minutes. The pellet was resuspended in 1 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitors. Cells were then sonicated using a 15 second on, 45 second off program 4 times consecutively; then diluted 1:10 in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH .1, 167 mM NaCl) plus protease inhibitor. The samples were then precleared overnight at 4°C with 75 µl Protein A/G Plus-agarose beads (sc-2003, Santa Cruz) supplemented with 3 µl 10 mg/ml Sonicated Salmon Sperm DNA (201190, Stratagene, La Jolla, CA) and 13 µl 1 mg/ml BSA. Samples were then incubated with 10 µg RNA Polymerase II (N-20) (sc-899, Santa Cruz) or the appropriate IgG overnight, tumbling at 4°C. Add 40 µl Protein A/G Plus-agarose beads for 2 hours rotating at 4°C. Samples were spun at 1000 rpm for 1 minute, then washed once with each: Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20
mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer. Samples were eluted twice in 50 µl in elution buffer (1% SDS, 0.1 M NaHCO₃) vortexing for 15 minutes at room temperature. 12 µl 5 M NaCl was added to eluate and incubated at 65°C overnight. Then 4 µl 0.5 M EDTA, 8 µl 1M Tris-HCl, pH 6.5 and 2 µl of 10 mg/ml proteinase K was added and incubated at 45°C for 1 hour. 

The samples were cleaned up using the QIAGEN PCR Clean up kit (QIAGEN). PCR was performed using TaKaRa Premix Ex Taq kit (RR039, TaKaRa, Otsu, Shiga, Japan). The final reaction contains: 1X Premix Ex Taq, 2.5 µM of each primer, 8% DMSO, and 20% processed DNA (or 20% of a 1:10 dilution of input). For amplification of GAPDH, forward: (5’-TACTAGCGGTTTTACGGGCG-3’) reverse: (5’-TCGAACAGGAGGAGCAGAGAGCGA-3’). For amplification of CDH11, forward: (5’-AAAGCAAAAGGGAGGGAGA-3’) reverse: (5’-AGGTACAAACCCCCTCTGCT-3’) primers were used. For amplification of PAI-1, forward: (5’-CAGAAAGGTTCAAGGGAGG-3’) and reverse: (5’-CCTGCAGCCAAACACACAGC-3’) were used.

III.C.VII. IMMUNOBLOTTING

Cells were treated for the indicated times then rinsed once with PBS and lysed with sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 7.5) containing 1 mM sodium orthovanadate, 0.05 M sodium fluoride, and Complete mini protease inhibitors
Cell lysates were boiled for 10 minutes. Protein concentration was determined with a Bio-Rad DC Protein Assay (500-0116, Bio-Rad, Hercules, CA). After SDS-poly acrylamide gel electrophoresis, proteins were transferred to Protran BA 83 Nitrocellulose (10402495, Germany). Membranes were blocked with 5% milk in Tris-Buffered Saline containing 0.1% Tween-20, and incubated with primary antibody overnight at 4°C and subsequently with HRP-labeled secondary antibody. Proteins were visualized with ECL chemiluminescent reagents (RPN2106, Amersham Biosciences, Piscataway, NJ) or SuperSignal West Femto (34095, Pierce biotechnology Inc., Rockford, IL) using X-ray film (Denville Scientific Inc., Metuchen, NJ).
III.D. RESULTS

III.D.I. TGFβ1 REGULATION OF CADHERIN-11

Cadherin-11 expressing breast cancer cells, MDA-MB-231 cells, were used as a cadherin-11 expression model in cancer (32). MDA-MB-231 cells, which do not express detectable levels of cadherins other than cadherin-11, were treated with TGFβ1 for the indicated length of time, and RNA isolated (94). Within 8 hours of TGFβ1 treatment, cadherin-11 RNA expression decreased and remained decreased as long as TGFβ1 is present (Figure 17A). PAI-1 is reported as a TGFβ1 responsive gene and is used as a positive control throughout these experiments (103). When TGFβ1 is added, PAI-1 expression increases within 8 hours and remains elevated as long as TGFβ1 is present (Figure 17A, right panel). Because PAI-1 expression is the converse of cadherin-11 expression, it is an appropriate positive control for treatment and cell responsiveness. Cadherin-11 protein expression resembles that of the RNA and also decreases with exposure to TGFβ1 (Figure 17B). At 72 hours, the signal returns suggesting the TGFβ1 signaling capability was exhausted.

I recently reported that cadherin-11 expression in MDA-MB-231 cells increases with cell density (see Chapter II). MDA-MB-231 cells were plated at three densities and serum-starved overnight. Cells were treated with TGFβ1 for 24 hours prior to RNA isolation for real-time PCR analysis (Figure 17C) and 48 hours prior to protein isolation for Western blot analysis (Figure 17D). At each density TGFβ1 attenuates cadherin-11 RNA expression and less robustly, the protein expression. TGFβ1 is also able to
increase PAI-1 expression showing both that the cells are responsive and that the TGFβ1 was effective (Figure 17C, right panel). Therefore, TGFβ1 treatment is able to partly overcome the increase in cadherin-11 expression due to increased density.

III.D.II. TGFβ1 REGULATION OF CADHERIN-11 IS NOT PRIMARILY TRANSCRIPTIONAL.

The conventional mechanism by which TGFβ1 regulates a gene is through transcriptional regulation. To determine whether TGFβ1 changes the rate of cadherin-11 transcriptional activation RNA was collected after treatment with actinomycin D alone or in concert with TGFβ1. Cells treated with either actinomycin D or TGFβ1 decreased cadherin-11 RNA expression at a similar rate suggesting a transcriptional mechanism of repression (Figure 18A).

To confirm that TGFβ1 regulates cadherin-11 in a transcriptional manner, anti-RNA Polymerase II (RNA Pol II) chromatin immunoprecipitation (ChIP) was used to determine whether the cadherin-11 gene is being transcriptionally regulated by TGFβ1. MDA-MB-231 cells were treated with TGFβ1 and/or actinomycin D for 24 hours, a time sufficient to decrease RNA levels, prior to harvesting DNA for ChIP processing (Figure 18B). Despite decreasing RNA levels after TGFβ1 treatment, and unlike treatment with actinomycin D, TGFβ1 fails to decrease the recruitment of RNA Pol II to cadherin-11.
III.D.III. NEITHER SMAD2 NOR SMAD3 HAVE AN EFFECT ON CADHERIN-11 EXPRESSION.

The TGFβ1 signal is usually transmitted through Smad2 and/or Smad3 phosphorylation. Therefore, if Smads 2 and 3 are necessary for the TGFβ1-dependent decrease in cadherin-11 expression, knock down of Smad2 or Smad3 should result in a change in cadherin-11 RNA and protein expression. Smad2 knock down was unable to prevent TGFβ1 from decreasing cadherin-11 protein (Figure 19A), and its loss actually decreases cadherin-11 protein in the absence of TGFβ1. Either knocking down or overexpressing Smad2 was unable to reverse the effects of TGFβ1 on cadherin-11 RNA expression (Figure 19B, upper panel). As expected, PAI-1 positively responds to TGFβ1 treatment positively (Figure 19B, lower panel).

Smad3 knockdown has little effect on cadherin-11 expression. Figure 19C shows a slight decrease in cadherin-11 protein expression 48 hours after siRNA transfection. As with Smad2 knockdown, Smad3 knockdown fails to prevent the TGFβ1-dependent decrease in cadherin-11 expression (Figure 19D). Furthermore, Smad3 knockdown was unable to reverse the effects of TGFβ1 treatment on cadherin-11 expression, and Smad3 overexpression was unable to mimic TGFβ1 repression (Figure 19E). In conclusion, neither Smad2 nor Smad3 play a role in TGFβ1 repression of cadherin-11.

III.D.IV. SMAD4 OVEREXPRESSAND KNOCKDOWN HAVE NO EFFECT ON CADHERIN-11 EXPRESSION.
Because Smads 2 and 3 are dependent on Smad4 for signal transduction, Smad4 was knocked down to ensure that Smad3 wasn’t compensating for Smad2 and vice versa. Smad4 knockdown had little effect on cadherin-11 protein expression (Figure 20A), and was unable to reverse the TGFβ1 decrease in cadherin-11 expression (Figure 20B). Furthermore, neither Smad4 knockdown nor overexpression was able to reverse the effects of cadherin-11 RNA repression (Figure 20C).

III.D.V. SMAD7 KNOCKDOWN DOES NOT CHANGE CADHERIN-11 EXPRESSION.

Recent studies have suggested that Smad7 may have roles other than that of a TGFβ pathway inhibitor. Therefore, I reasoned that Smad7 knockdown may have an effect on cadherin-11 expression. In the absence of Smad7 without TGFβ1 treatment, cadherin-11 levels remain relatively constant. As expected, treatment with TGFβ1 dramatically increased levels of Smad 7; however Smad7 knockdown is unable to reverse the effect of TGFβ1 on cadherin-11 expression (Figure 21A). Further, cadherin-11 RNA is unaffected by the absence of Smad7 and continued to decrease in the presence of TGFβ1 (Figure 21B). As expected, Smad7 knockdown significantly increased PAI-1 expression even in the absence of TGFβ1 treatment (Figure 21B, lower panel).

III.D.VI. SMAD-INDEPENDENT TGFβ1 PATHWAY INHIBITION AFFECTS CADHERIN-11 EXPRESSION.
TGFβ1 is known to activate pathways independently of Smad2/3 signaling. The pathways I investigated include p38 MAPK, JNK, MEK1, and TGFβ1 type 1 receptor. Two small molecule inhibitors caused a change in cadherin-11 RNA expression. p38 inhibition (SB-202190) mimicked the effect of TGFβ1 on cadherin-11 (Figure 22A and 22B), and TGFβ1 type 1 receptor (TβRI) inhibition (SB-431542) blocked the effect of TGFβ1 on cadherin-11 entirely (Figure 22). Furthermore, inhibition of either of these pathways decreased PAI-1 activation in response to TGFβ1 (Figure 22A, lower panel). Finally, inhibition of either p38 MAPK or TβRI blocked the decrease in cadherin-11 protein expression due to TGFβ1 treatment (Figure 22B). Therefore, TβRI and p38 MAPK are important to cadherin-11 regulation.

III.D.VII. p38 MAPK KNOCKDOWN MIMICKED TGFβ1 TREATMENT WITH RESPECT TO CADHERIN-11 EXPRESSION.

Several isoforms of p38 MAPK are known to be expressed in MDA-MB-231 cells. SB-202190 inhibits p38α and p38β with residual effects on p38γ and minimal effects on p38δ. Therefore I began my investigation by focusing on p38α since MDA-MB-231 cells express low levels of p38β. Knockdown of p38α MAPK (MAPK14) decreased cadherin-11 RNA expression mimicking TGFβ1 and SB-202190 treatments. No change in cadherin-11 RNA expression was observed in cells treated with TGFβ1 or cells treated with siRNA directed against MAPK14 (Figure 23). SB-202190, TGFβ1, and siRNA directed against p38α have similar effects with respect to cadherin-11 knock
down. MAPK14 knockdown reduces cadherin-11 protein in a manner similar to that of TGFβ1 (Figure 23C). Cells treated with either TGFβ1, SB-202190, or MAPK14 siRNA all had less cadherin-11 expression than DMSO (control) treated cells. p38 inhibition or knockdown mimicked TGFβ1 treatment suggesting that p38 is yet another mechanism by which cadherin-11 is regulated, although most likely, independent of TGFβ1.

III.D.VIII. TGFβ1 ACTS THROUGH THE TGFβ1 TYPE I RECEPTOR TO REDUCE CADHERIN-11 EXPRESSION.

SB-431542 blocks all Smad-dependent and Smad-independent TGFβ1 signaling pathways that rely on TGFβ type I receptor kinase activity (47). MDA-MB-231 cancer cells were treated with SB-431542 in the presence and absence of TGFβ1. Cells treated with TGFβ1 decreased cadherin-11 RNA levels, as seen in Figure 17A. In contrast, cells treated with SB-431542, either alone or in concert with TGFβ1, failed to decrease cadherin-11 expression, resembling the untreated control (Figure 24A). Therefore, the TGFβ1-dependent decrease in cadherin-11 expression is transmitted through the TGFβ1 receptor.

To determine whether TGFβ1 permanently alters the cadherin-11 promoter, SB-431542 was used to block TGFβ1 signaling 24 hours after TGFβ1 treatment. MDA-MB-231 cells were treated with TGFβ1 for 24 hours before treatment with SB-431542 at time 0 to “wash out” the effect of TGFβ1. RNA was collected at the specified times. Cadherin-11 expression was decreased at time 0, consistent with Figures 17 and 18A,
but 8 hours after the addition of SB-431542, cadherin-11 levels began to recover reaching control levels within 24 hours of treatment (Figure 24B). Thus, the effect of TGFβ1 is reversible with respect to cadherin-11 expression.

III.E. DISCUSSION

There are three very similar TGFβ signaling molecules, but TGFβ1 is most frequently upregulated in tumor cells (18). Activation of TGFβ1 in tumor cells effects the local environment in several ways. First of all, the tumor breaks down the stroma by inducing the expression of metalloproteases MMP-2 and MMP-9 and suppressing TIMP-1 (27). Then TGFβ1 activates the expression of VEGF, molecules which stimulate endothelial cells and begins the process of angiogenesis (90). Angiogenesis allows the tumor to not only invade the local tissue, but ultimately the organism. Finally, TGFβ1 expression by the tumor cells suppresses the local immune function, increasing overall tumorigenicity (121). Patients with elevated serum levels of TGFβ1 generally have a poor prognosis (49).

TGFβ1 also plays a role in the progression of arthritis. It is associated with inflammation, one of the factors commonly thought to provoke arthritis, and angiogenesis. It has been suggested that TGFβ1 may even play a role in osteophyte formation (124).

Two reports show that TGFβ1 increased cadherin-11: first in cytotrophoblast cells and second in primary human kidney tubular cells (31, 32). Our unpublished work
also shows that unlike MDA-MB-231 cells several other cell lines increase cadherin-11 levels in response to TGFβ1. Other reports examined the TGFβ1 response in cadherin-11-expressing cells lines without reporting an outcome with respect to cadherin-11 (27). Cadherin-11 is often expressed by invasive metastatic tumors and cell lines, and TGFβ1 is also expressed and often misregulated in these samples.

The best-studied TGFβ1 mechanism is that of the Smad signaling pathway, which classically regulates gene expression though a transcriptional mechanism. The decrease in cadherin-11 expression with TGFβ1 treatment strongly suggested a transcriptional deactivating event, such as activation of Snail, a TGFβ1 target. I eliminated components of this pathway either singly or together (data not shown), using RNAi, to reveal that Smad signaling has no significant effect on cadherin-11 expression. ChIP analysis provided further evidence that TGFβ1 does not decrease cadherin-11 transcription. Other Smad-independent pathways are activated by TGFβ1; p38 MAPK is of particular interest. p38 MAPK downstream targets are known to play a role in RNA stability, a possible mechanism of cadherin-11 regulation (98). p38 knockdown, inhibition, or TGFβ1-dependent activation caused a reduction in cadherin-11 expression suggesting p38 functions independently of TGFβ1 signaling. In MDA-MB-231 cells, TGFβ1 activates p38 within one hour of treatment, which is not sustained throughout the 24 hour treatment (102). Because all of our treatments have been collected after 24 hours, I may be observing an effect independent of p38 activation by TGFβ1. Other inhibitor experiments using SB-431542 show that the
changes in cadherin-11 expression are certainly transmitted through the traditional TGFβ1 receptor.

TGFβ1 mechanisms have been well-studied, and p38 MAPK is a well-known downstream target of the TGFβ1 pathway. Cadherin-11 is known to be regulated by TGFβ1 during both development and cancer in other model systems. Although several reports link the two proteins, none look at the intermediate steps necessary for regulation. This report elucidates the pathway components and their effect on cadherin-11 regulation in MDA-MB-231 cells.

III.F. ACKNOWLEDGEMENTS

This work was supported by DOD Idea Award DAMD17-01-1-0245 (SWB) and DOD Prostate Cancer Research Predoctoral Traineeship Award W81XWH-05-1-0559 (AKF).

III.G. FIGURE LEGENDS

FIGURE 17: TGFβ1 TREATMENT DECREASES CADHERIN-11 EXPRESSION IN CANCER CELLS.

A and B: MDA-MB-231 cells were plated at a medium density and serum-starved overnight. Cells were treated with 5 ng/ml TGFβ1, and RNA was collected at the indicated time points for real-time PCR (A) or Western blot (B) analysis. C and D: MDA-MB-231 cells were plated at three densities, 50%, 80%, and 90% confluency, called low, medium, and high, respectively. The cells were allowed to grow overnight in serum-free medium. The cells were untreated (U) or treated with 5 ng/ml TGFβ1 (T) for 24 hours at which time RNA was collected for real-time PCR (C) and Western blot
Figure 18: A transcriptional mechanism of regulation. A: MDA-MB-231 cells were pretreated for 30 minutes with 5 μg/ml actinomycin D or an equivalent volume of ethanol (untreated), after which the cells were treated with 5 ng/ml TGFβ1. At the indicated time points, RNA was collected for real-time PCR analysis. B: MDA-MB-231 cells were untreated or treated with 10 ng/μl TGFβ1 for 24 hours. Genomic DNA was then harvested for RNA polymerase II ChIP analysis followed by PCR specific to GAPDH, cadherin-11, and PAI-1. Graphs are a compilation of experiments; error bars depict standard error of the mean (SEM). (* represents p < 0.05).

Figure 19: Cadherin-11 expression in the presence and absence of Smad2 and Smad3. A: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad2 (siSmad2). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. Protein was isolated for Western blot analysis. B: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble), siRNA directed against Smad2 (siSmad2), pcDNA3, or wild type Smad2 (wt Smad2). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. RNA was collected for real-time PCR analysis. C: MDA-MB-231 cells were
transfected with non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad3 (siSmad3). At the specified time points, protein was collected for Western blot analysis. D: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad3 (siSmad3). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. Protein was isolated for Western blot analysis. E: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble), siRNA directed against Smad3 (siSmad3), pcDNA3, or wild type Smad3 (wt Smad3). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. RNA was collected for real-time PCR anaylsis.

**FIGURE 20: CADHERIN-11 EXPRESSION IN THE PRESENCE AND ABSENCE OF SMAD4. A:** MDA-MB-231 cells were transfected with non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad4 (siSmad4). At the specified time points, protein was collected for Western blot analysis. B: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble) and siRNA directed against Smad4 (siSmad4). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. Protein was isolated for Western blot analysis. C: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble), siRNA directed against Smad4 (siSmad4), pcDNA3, or wild type Smad4 (wt Smad4). 24 hours after transfection, the cells were
treated with 10 ng/ml TGFβ1 for an additional 24 hours. RNA was collected for real-time PCR analysis. Histograms are a compilation of experiments; error bars depict standard error of the mean (SEM). (* represents p < 0.05)

**Figure 21:** Cadherin-11 expression in the presence and absence of Smad7. A: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad7 (siSmad7). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. Protein was isolated for Western blot analysis. B: MDA-MB-231 cells were transfected with either non-specific scrambled sequence siRNA (siScramble) or siRNA directed against Smad7 (siSmad7). 24 hours after transfection, the cells were treated with 10 ng/ml TGFβ1 for an additional 24 hours. RNA was isolated for real-time PCR analysis.

**Figure 22:** TGFβ1 pathway inhibitors affect Cadherin-11 expression. A and B: MDA-MB-231 cells were plated at a medium density and serum starved overnight. Cells were pretreated with 10 µM of the specified inhibitor for 30 minutes prior to the addition of 10 ng/ml of TGFβ1. Samples were collected 24 hours after treatment for real-time PCR analysis (A) and 48 hours after treatment for Western Blot analysis (B). Histogram is a compilation of experiments; error bars depict standard error of the mean (SEM). (* represents p < 0.05)
Figure 23: p38α MAPK knockdown is unable to prevent TGFβ1 suppression of cadherin-11. A and B: MDA-MB-231 cells were transfected with either non-specific scrambled siRNA (siScramble) or siRNA directed against p38α MAPK (si p38). 24 hours after transfection, the cells were treated with DMSO, 10 ng/µl TGFβ1, 10 µM SB-202190, or 10 ng/µl TGFβ1 and 10 µM SB-202190 for an additional 24 hours. RNA was collected for real-time PCR (A) and Western blot (B) analysis. Histogram is a compilation of experiments; error bars depict standard error of the mean (SEM).

Figure 24: Inhibition of the TGFβ1 type I receptor is sufficient to block the TGFβ1-dependent decrease in cadherin-11 expression. A: MDA-MB-231 cells were plated at a medium density and serum starved overnight. Cells were pretreated with 10 µM SB-431542 for 30 minutes prior to the addition of 5 ng/ml of TGFβ1. Samples were collected at the indicated times for real-time PCR analysis. B: MDA-MB-231 cells were plated at a medium density, serum starved overnight, and treated with 5 ng/ml TGFβ1 for 24 hours at which point (time 0) 10 µM SB-431542 was added to the designated samples. RNA was collected at the times indicated for real-time PCR analysis.
III.H. FIGURES

FIGURE 17. TGFβ1 TREATMENT DECREASES CADHERIN-11 EXPRESSION IN CANCER CELLS
C

![Graph showing the expression levels of CDH11 and PAI-1 RNA for different cell densities and treatments.](image)

D

![Western blot image showing CDH11 and α-tubulin expression levels for different cell densities and treatments.](image)
FIGURE 18. A TRANSCRIPTIONAL MECHANISM OF REGULATION

A

![Graph showing changes in CDH11 and PAI-1 RNA levels over time under different treatments.]

B

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<tr>
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<td>PAI-1</td>
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* denotes significant difference from untreated control.
Figure 19. Cadherin-11 expression in the presence and absence of Smad2 and Smad3

A

B

C

D

E
FIGURE 20. CADHERIN-11 EXPRESSION IN THE PRESENCE AND ABSENCE OF SMAD4

A

B

C

CDH11

Smad4

GAPDH

CDH11

Smad4

GAPDH

CDH11 RNA [Fold Change]

CDH11 RNA [Fold Change]

PAI-1 RNA [Fold Change]

Untreated

TGFb1

Untreated

TGFb1

Untreated

TGFb1
Figure 21. Cadherin-11 expression in the presence and absence of Smad7

A

B

CDH11 RNA [Fold Change]

0
0.2
0.4
0.6
0.8
1
1.2
Untreated
Tb1

CDH11 RNA [Fold Change]

0
0.2
0.4
0.6
0.8
1
1.2
Untreated
Tb1
FIGURE 22. TGFβ1 pathway inhibitors affect cadherin-11 expression

A

![Bar graph showing CDH11 RNA expression](image)

B

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CDH11

Hsp 72/73
Figure 23. p38 MAPK knockdown is unable to prevent TGFβ1 suppression of cadherin-11.
Figure 24. Inhibition of the TGFβ1 type I receptor is sufficient to block the TGFβ1-dependent decrease in cadherin-11 expression.
CHAPTER IV
CONCLUSIONS AND DISCUSSION

This body of work set out to define the mechanisms that regulate cadherin-11 expression. It is known that cadherin-11 is expressed by invasive metastatic cancers of the breast and prostate specifically. It was the hope of this study to determine what changes within the cell trigger cadherin-11 expression. I have found that density, GSK3 inhibition, and TGFβ1 effect changes in cadherin-11 expression. Density profoundly increases cadherin-11 expression in MDA-MB-231 breast cancer cells an effect that is reversed by either GSK3 inhibition or TGFβ1 treatment. Both GSK3 inhibition and TGFβ1 treatment decrease cadherin-11 in a dose- (data not shown) and time-dependent manner in MDA-MB-231 cells. GSK3 inhibition has β-catenin-dependent and β-catenin-independent mechanisms to regulate protein alone or protein and mRNA, respectively. The means by which TGFβ1 affects cadherin-11 relies on signal transmission through the TGFβ1 type I receptor. Additionally, p38 acts independently of TGFβ1 to regulate cadherin-11 expression. Figure 25 schematically summarizes these data.

IV.A. CADHERIN REGULATION BY CELL DENSITY/CONFLUENCE

The basis of my interest in cell density began with a literature search investigating how density affects other cadherins. Conacci-Sorrell et al report that E-cadherin was regulated in a density-dependent manner (13). They demonstrate that E-cadherin expression levels are increased by high cell density in SW480 cells, a colon
Because E-cadherin is known to be expressed by epithelial-like, non-motile cells, I was interested in comparing cadherin-11 expression with respect to density to both an epithelial and a mesenchymal cadherin. Wanner and Wood found changes in the mesenchymal cadherin, N-cadherin, expression with changing density. They found that increased cell density resulted in a decrease in N-cadherin protein levels in rat Schwann cells suggesting that N-cadherin plays a pertinent adhesive role in mesenchymal cells (126).

Feltes et al. showed that when minimally motile cells were transfected with cadherin-11 in concert with cadherin-11 variant they had increased motility and invasiveness (28). This report concluded cadherin-11 and cadherin-11 variant together have a positive contribution to cell motility. Because of this work and others, cadherin-11 has been classified as a mesenchymal cadherin and often compared to N-cadherin. Therefore, we were surprised when cadherin-11 expression increased with increasing cell density (Figure 7A and 7B), like E-cadherin not N-cadherin. Two possible explanations as to why cadherin-11, a mesenchymal cadherin, is regulated by density like an epithelial cadherin are: 1) cadherin-11 was initially misclassified and 2) each cadherin-11 has its own mechanism of regulation and should not be compared to those it resembles in function. Explanation 1 is unlikely. Explanation 2 is explored in Table 1, which implies that the cadherin-11 3’UTR is conserved as a regulatory mechanism. Although the UTRs of other cadherins may be important for regulation, those sequences have very little identity to the equivalent sequence of cadherin-11 and each other (data
not shown). Therefore, although cadherins may resemble each other in structure and function, they are dissimilar with respect to post-transcriptional regulation.

IV.B. REGULATION BY RNA STABILITY

Recently an article detailed how the combination of *cis*-elements in the 3’-UTR affects translational timing. This report explores the 3’UTRs of the *Xenopus* cyclin B genes in depth to determine why each gene is translated at a specific timepoint. Pique *et al* found that the organization of cytoplasmic polyadenylation elements (CPEs) and pumilio binding elements (PBEs) affected the timing and rate of translation through 3’UTR poly-adenylation (93). This combinatorial mechanism of translational regulation strongly resembles the complex mechanism necessary for transcriptional initiation. Certain elements are absolutely necessary, but other regulatory factors determine whether translation can initiate. In addition to an extensive report, Pique *et al* also published their web-based computational algorithm. Analysis of the cadherin-11 3’UTR for these specific *cis*-elements reveals many putative CPEs, but none of the consensus PBEs. The computational program concluded that, if occupied, these elements in this arrangement would act to repress cadherin-11 mRNA translation.

Cadherin-11, if regulated by cytoplasmic polyadenylation element binding proteins (CBEPs), would be expected to be repressed in the presence of this binding protein (93). This algorithm was designed in *Xenopus* and applied to mammalian genes. Although pumilio and CBEP are conserved throughout evolution and are
expressed in mammalian cells, the *cis*-binding sequences may differ. Mutation of these CPEs, addition of a PBE upstream of the hexamer, or knockdown of CPEB may confirm that cadherin-11 is being repressed by this mechanism. Additionally, the Puf repeat region of Pumilio bears a striking resemblance to the armadillo repeats of β-catenin suggesting that they may function similarly.

Another common mechanism of 3’UTR regulation involves *trans*-factor binding to AU-rich elements (AREs). The list of proteins that bind to AREs continues to grow and includes both stabilizing and destabilizing proteins. HuR and β-catenin are a well-known ARE-binding proteins that stabilize RNA, but other ARE-interacting proteins, tristetraprolin (TTP) and T-cell restricted intracellular antigen (TIA)-1, play a role in destabilizing mRNAs. In the future directions of this project, I suggest emphasizing the role AREs play in regulation of cadherin-11. One methodology would involve mutation or deletion of these sequences from CDH11 3’UTR Long, the more unstable construct. Additionally, TTP is known to be upregulated by TGFβ1 but no association has been made with Wnt pathway members; there are no reports of TGFβ1 or Wnt pathway interaction with TIA-1. Other experiments may include overexpressing a dominant negative ARE-binding protein, that blocks the *cis*-element, preventing downregulation. ARE-binding proteins encompass a considerable number of proteins any of which is a possible cadherin-11 3’UTR interacting protein. I believe this topic will definitely be involved in the future work relating to cadherin-11 RNA stability.
Regulation by miRNAs is yet another mechanism by which 3’UTRs are regulated. miRNAs affect both degradation and translational repression of mRNAs, but seem to have a modest effect on cadherin-11 RNA stability, as seen by Dicer knockdown. There are many more putative miRNA binding sites in the cadherin-11 3’UTR than were individually explored, but as these additional miRNAs would all depend on Dicer expression, I believe that this is not the primary mechanism of regulation.

The process of translating RNA requires a delicate balance of elements. Figure 26 depicts the relationships of above mechanisms and the expected outcome. CPEB and Pumilio are involved in translational repression and activation. ARE-binding proteins, such as HuR and TTP, usually affect RNA stability through either stabilization and increased translation, destabilization and translational repression, or RNA degradation. Finally, miRNA regulation typically leads to sequestration in processing-bodies (P-bodies) leading to translational repression or RISC complex association and degradation. Reports have found miRNAs to be bound to mRNAs associated with polysomes, but these are thought to effect translational derepression.

IV.C. Wnt SIGNALING PROTEINS AND CADHERINS

Intrinsically cadherins rely on β-catenin for stability. Mature adherens junctions only form when several events occur: the extracellular cadherin domains must be rigid and have Ca\(^{2+}\) bound, the cadherin domains of two opposing cells must interact, and the
intracellular domain must bind armadillo-like proteins (β-catenin, p120ctn, γ-catenin). In some cases one armadillo-like protein will substitute for another to stabilize adherens junctions. Because β-catenin plays multiple roles in this set of circumstances, one of stabilizing the protein at the membrane and another though intracellular signaling, the story becomes complicated and sometimes difficult to decipher.

Treatment of cancer cells with either LiCl or BIO leads to cellular accumulation and stabilization of β-catenin and a decrease in cadherin-11 expression. Does the decrease in cadherin-11 expression lead to β-catenin accumulation? or Does inhibiting the degradation pathway cause β-catenin accumulation? Either way, my interest is in the decrease in cadherin-11 and determining whether this decline is caused by the accumulation of β-catenin. When β-catenin was knocked down in PC-3 cells, BIO was less effective at decreasing cadherin-11 as compared to control (Figure 10C). As described previously, PC-3 cells and MDA-MB-231 cells are fundamentally different with respect to β-catenin accumulation and regulation. PC-3 cells have a PTEN mutation which leads to increased cellular β-catenin and decreased GSK3 function. MDA-MB-231 cells have normal GSK3 function but low levels of intracellular β-catenin. These two cellular model systems side by side are useful for examination of how this pathway interacts with cadherin-11. BIO treatment in MDA-MB-231 cells results in a cadherin-11 bandshift, which was not observed in cells treated with CTNNB1 siRNA. Surprisingly, knock down or overexpression of β-catenin in either of these cells lines did not change the effect of BIO on cadherin-11 RNA. Therefore, BIO
acts through β-catenin to decrease cadherin-11 protein expression, but another mechanism exists, which is not explored here, by which BIO decreases RNA expression.

The story about GSK3 and cadherin-11 or β-catenin and cadherin-11 is far from complete. β-catenin was proposed to be involved in RNA stability of VEGF-D (89). More recently, β-catenin was shown to bind and stabilize COX-2 RNA (65). The possibility that β-catenin binds to the cadherin-11 mRNA is promising and is yet to be determined.

IV.D. CADHERIN-11 AND TGFβ1

My interest in cadherin-11 regarding TGFβ1 came about because the cells of the extravillious cytotrophoblast responded to TGFβ1 treatment by increasing cadherin-11 expression (32). Furthermore, both are expressed in late stage cancer. MDA-MB-231 cells respond to TGFβ1 and metastasize to the bone in response to this cytokine (27, 130). In addition to the effects on bone metastasis, cadherin-11 and TGFβ1 both play a role in the joint and development of arthritis. Cadherin-11 is necessary for the formation of the destructive pannus, and TGFβ1 pathway components are upregulated in arthritic tissue (63, 95). Expression in the same place at the same time suggests that cadherin-11 and TGFβ1 are possibly partners in crime.

In most cases where cadherin-11 is expressed, TGFβ1 is suspected to also be involved. Several reports suggest that when TGFβ1 activity is high, cadherin-11 is
expressed, whether that is in the extravillous cytotrophoblast, the synovium, or the bone (32, 39, 53, 95, 122). However, in MDA-MB-231 cells TGFβ1 has the opposite effect; cadherin-11 expression is downregulated in response to TGFβ1 treatment. A previous report looked at expression of TGFβ1 pathway components in MDA-MB-231 cells. Smads 2, 3, 4 and both TGFβ receptors type I and type II were expressed (97). In fact, of the cells examined, MDA-MB-231 cells were the only cell line to show induction of the PAI-1 promoter, a benchmark for TGFβ1 responsiveness (97). Farina et al (no relation) found that TGFβ1 treatment increased the invasiveness of MDA-MB-231 cells, through upregulation of urokinase (27). Although they failed to explore cadherin-11 expression, I assume that in addition to increased urokinase activity, cadherin-11 expression was probably decreased. To confound the story, preliminary data has shown that knock-down of cadherin-11 in MDA-MB-231 cells successfully decreased the invasive potential of the cells as determined by both Matrigel outgrowth and Boyden chamber assays (data not shown). In addition, expression of cadherin-11 alone decreased the ability of SkBr3 cells to invade, but cadherin-11 expression in conjunction with cadherin-11 variant significantly increased cell motility (28). Perhaps TGFβ1 repression of cadherin-11 temporarily allowed the cells to detach from each other, promoting motility. In conclusion, TGFβ1 undeniably has an effect on cadherin-11 expression and increases cell motility. Unfortunately, the reports remain ambiguous.

IV.E. CONCLUSION
Cadherin-11 plays a role in both development and disease progression. Reports indicate that compounds other than Wnt and TGFβ1, such as curcumin, 3,3'-diindolylmethane (DIM), progesterone, and TGFα have effects on cadherin-11 expression (9, 80, 133). I believe that cadherin-11 research will broaden from a cancer and development focus to include an arthritis focus. In cancer, cadherin-11 is expressed by the most invasive and metastatic tumors, which isn’t determined until it is too late for anti-cadherin-11 treatment to have any effect. In the case of arthritis, anti-cadherin-11 therapy can possibly help before the damage is irreparable. Anti-cadherin-11 treatments have yet to be tested in extreme models of arthritis, but the future looks promising. As to the regulation of cadherin-11, even in fibroblast-like synoviocytes, I suspect RNA stability will become a significant part of this story.

IV.F. FIGURE LEGENDS

FIGURE 25. SCHEMATIC SUMMARY OF DATA

FIGURE 26. SCHEMATIC DEPICTION OF MECHANISMS REGULATING TRANSLATIONAL CONTROL
IV.G. FIGURES

FIGURE 25. SCHEMATIC SUMMARY OF DATA
Figure 26. Schematic depiction of the mechanisms regulating translational control.
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