The Embryonic Protein Nodal Supports Metastatic Phenotypes in Breast Cancer

Daniela F. Quail
The University of Western Ontario

Supervisor
Dr. Lynne-Marie Postovit
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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THE EMBRYONIC PROTEIN NODAL SUPPORTS METASTATIC PHENOTYPES IN BREAST CANCER

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by

Daniela F. Quail

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
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ABSTRACT

Metastasis is the process by which tumour cells disseminate to distant organ sites. Aberrant expression of stem cell-associated proteins within tumours is associated with metastasis and poor patient prognosis. One example of a stem cell factor that is associated with cancer progression is Nodal, a member of the TGF-β superfamily. Nodal is normally limited to pluripotent stem cells during embryonic development, and to specialized dynamic adult tissue (such as the cycling endometrium), but is aberrantly re-expressed in multiple cancer types, including melanoma, glioma, prostate cancer, and pancreatic cancer. The central objective of this thesis is to determine the role of Nodal during various aspects of the metastatic cascade in breast cancer. First, I determined that Nodal inhibition in aggressive breast cancer cell lines impairs tumour growth in an orthotopic nude mouse model, concomitant with reduced proliferation and enhanced apoptosis. Furthermore, in an experimental metastasis assay in NOD/SCID/MPSVII mice, I determined that Nodal knockdown prevents the transition from lung micrometastases to macrometastases, by supporting a positive ratio of proliferation to apoptosis. Using numerous animal models, I then discovered that Nodal promotes angiogenesis, and that knocking down its expression in established tumours reduces vascularization and causes necrosis. Notably, Nodal protein was positively correlated with vascular density in human breast cancer lesions. Mechanistically, Nodal induced a pro-angiogenic profile in breast cancer cells by upregulating VEGF and PDGF. Finally, I investigated the role of Nodal in the
regulation of EMT and invasion; phenotypes that are classically associated with this morphogen. Specifically, since Nodal is implicated in mammary gland remodeling and placentation, I examined its effects on cellular invasion in these contexts. Nodal overexpression in poorly metastatic breast cancer and choriocarcinoma cell lines enhanced invasion and EMT-associated changes in gene expression, and this effect was in part mediated by ERK signaling. Nodal inhibition in metastatic breast cancer cell lines reduced spontaneous metastasis to the liver (but not the lung) in NOD/SCID/IL2γR- mice. The results presented herein suggest that Nodal promotes several pro-metastatic processes. Given its restriction to embryonic or highly specialized adult contexts, targeting Nodal in breast cancer poses an exciting avenue for therapeutic intervention.

KEYWORDS

Nodal, Transforming Growth Factor-beta (TGF-β), breast cancer, proliferation, apoptosis, macrometastasis, angiogenesis, angiogenic switch, endothelial cell, vascularization, invasion, migration, epithelial-to-mesenchymal transition (EMT), E-Cadherin, TWIST1, estrogen receptor (ESR1), metastasis
If you’re out to describe the truth, leave elegance to the tailor.

- Albert Einstein
DEDICATION

To my grandfather, F. James Quail, whose love for science was contagious.

To my nonna, Maria Allevato, who won her battle with breast cancer. Alla mia nonna, Maria Allevato, che ha sormontato il cancro della mammilla.

Finally, to my father, Peter R. Quail, who is the reason I started this journey in the first place.
STATEMENT OF CO-AUTHORSHIP

Each chapter constitutes a manuscript that has been submitted or accepted to a peer-reviewed journal. Contributions from all authors are as follows:

**Chapter 2: Nodal inhibition impairs breast cancer growth and progression**

Chapter 2 was submitted to PLoS ONE for publication as a Standard Research Article. In order, authors include Quail DF, Zhang G, Walsh LA, Broughton H, Hess DA, and Postovit LM. GZ helped with all *in vivo* tumour assays, and performed staining for TUNEL, Ki67, and H&E. LAW made the Nodal expression construct, and helped generate stable cell lines. HB performed all tail vein injections in NOD/SCID/MPSVII mice, and performed GUSB staining on tissue. DAH and LMP contributed to experimental design, and interpretation of results throughout the manuscript. As first author, I performed and/or oversaw all experiments pertaining to this study, analysed and interpreted the results, and wrote the manuscript.

**Chapter 3: The embryonic protein Nodal promotes breast cancer vascularization**

Chapter 3 was submitted to Cancer Research for publication as a Research Article. In order, authors on this manuscript include Quail DF, Walsh LA, Zhang G, Findlay S, Fung L, Ablack A, Moreno J, Lewis J, Done S, Hess DA, and Postovit LM. LAW made the Nodal expression construct, helped generate stable cell lines, helped with endothelial cell assays, and helped with mouse experiments. GZ helped with mouse experiments, and performed all IHC. SF helped with analysis of *VEGF* expression in tumour tissue. LF, AA, and JL performed the CAM assays and CAM image acquisition. JM and SD performed all scoring and analysis of Nodal and CD31 in human tissue
sections. LMP described and analyzed the clinical characteristics of the 83 human tumour samples that were obtained from the OICR. DAH and LMP contributed to experimental design and interpretation of results throughout the manuscript, and helped write the manuscript. As first author, I performed and/or oversaw all experiments pertaining to this study, analysed and interpreted the results, and wrote the manuscript.

Chapter 4: Nodal promotes invasion phenotypes via a non-canonical Mitogen Activated Protein Kinase-dependent pathway

Chapter 4 was submitted to Oncogene for publication as an Original Article. In order, authors on this manuscript include Quail DF, Zhang G, Hess DA, and Postovit LM. GZ helped with all mouse experiments, and contributed substantially to the choriocarcinoma portion of the manuscript. DAH and LMP contributed to experimental design and interpretation of results throughout the manuscript, and helped write the manuscript. As first author, I performed and/or oversaw all experiments pertaining to this study, analysed and interpreted the results, and wrote the manuscript.
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LIST OF ABBREVIATIONS

BMP ................................................................. Bone morphogenetic protein
FGF ............................................................... Fibroblast growth factor
ECM .............................................................. Extracellular matrix
ESA ............................................................... Epithelial-specific antigen
UDH ............................................................. Usual ductal hyperplasia
ADH ............................................................. Atypical ductal hyperplasia
DCIS ............................................................. Ductal carcinoma in situ
IDC ............................................................... Invasive ductal carcinoma
ER ................................................................. Estrogen receptor
PR ................................................................. Progesterone receptor
APC/C .......................................................... Anaphase -promoting complex/cyclosome
CDK ............................................................. Cyclin-dependent kinase
EGFR ......................................................... Epidermal growth factor receptor
PARP-1 ....................................................... Poly-ADP-ribose polymerase-1
AIF .............................................................. Apoptosis inducing factor
NO ............................................................... Nitric oxide
IAP ............................................................... Inhibitor of apoptosis protein
SMAC ...................................................... Second mitochondria-derived activator of caspases
MAC ........................................................ Mitochondrial apoptosis-induced channel
APAF1 ......................................................... Apoptotic protease activating factor-1
TNF ................................................................. Tumour necrosis factor
TRADD ......................................................... TNF receptor-associated death domain
BMDC ................................................................. Bone marrow-derived cell
VEGF ................................................................. Vascular endothelial growth factor
HIF ................................................................. Hypoxia inducible factor
MMP ................................................................. Matrix metalloproteinase
PDGF ................................................................. Platelet-derived growth factor
EMT ................................................................. Epithelial-to-mesenchymal transition
IGF-1 ................................................................. Insulin-like growth factor-1
EndMT ................................................................. Endothelial-to-mesenchymal transition
TGF-β ................................................................. Transforming growth factor-beta
CAF ................................................................. Cancer-associated fibroblast
ALK ................................................................. Activin-like kinase
FOXH1 ................................................................. Forkhead Box H1
CER1 ................................................................. Cerberus-like
LSE ................................................................. Left side-specific enhancer
ASE ................................................................. Asymmetric enhancer
NDE ................................................................. Node-specific enhancer
SPC ................................................................. Subtilisin-like proprotein convertase
GDF1 ................................................................. Growth differentiation factor 1
EBAF ................................................................. Endometrial bleeding-associated factor (a.k.a. Lefty)
CSC ................................................................. Cancer stem cell
GUSB ................................................................. Beta-glucuronidase
MPSVII .............................................................. Mucopolysaccharidosis type VII
MVD ................................................................. Microvascular density
SDF-1 ................................................................. Stromal-derived factor-1
HUVEC ............................................................. Human umbilical cord vein endothelial cells
HMVEC ............................................................. Human adult microvascular endothelial cells
DIVAA .............................................................. Directed in vivo angiogenesis assay
CAM ................................................................. Chick chorioallantoic membrane
Dox ................................................................. Doxycyclin
BMD ................................................................. Bone marrow-derived
IL2γR ............................................................... Interleukin-2-gamma receptor
rhNodal ............................................................ Recombinant human Nodal
NSG ................................................................. NOD/SCID/IL2γR-negative
ZAG ................................................................. Zinc-alpha 2-glycoprotein
EPC ................................................................. Endothelial progenitor cell
MMTV ............................................................. Mouse mammary tumour virus
PyMT ............................................................... Polyoma middle T-antigen
MET ............................................................... Mesenchymal-to-epithelial transition
TAM ............................................................... Tumour-associated macrophage
CHAPTER 1

General Introduction and Literature Review
1.0 General Introduction and Literature Review

In Canada, 40% of women and 45% of men will develop cancer during their lifetimes. Unimaginably, 1/4 Canadians will die from cancer or a cancer-related death (1). Unfortunately, cancer is not a single disease; rather, it is a dynamic orchestrated phenomenon that involves multiple divergent cell types, communication between cells and their microenvironments, rearrangement and turnover of extracellular components, genomic instability and epigenetic alterations, and complex signalling networks between primary and pre-secondary sites (2-5). Furthermore, just as breast cancer is wildly divergent from a brain tumour, breast cancer in one patient behaves and arises differently than breast cancer in another patient (6-8), rendering the fight for the “cure” difficult to resolve. Accordingly, research geared towards elucidating the intricate molecular mechanisms that regulate the initiation and progression of this multifaceted disease is imperative in order to develop strategies for improved patient outcome.

Breast cancer, in particular, directly afflicts one-in-nine Canadian women (1). The cause of death during breast cancer progression is primarily metastasis to secondary organs, most often involving liver, lung and bone. Consequently, understanding the mechanisms that underlie the transition from local to metastatic disease is of interest to both cancer scientists and the general public, and is the central focus of this thesis.
1.1 Normal breast anatomy and development

The mammary gland is a complex network of cell types and microenvironmental factors, which mediate lactation and transfer of milk, fat and protein between a mother and infant. Our knowledge of mammary gland development in humans is largely based on observations of mammary gland development in mice. In both rodents and humans, there are three key phases of mammary gland development, during embryonic development, puberty, and reproductive life (9,10):

In mice, the first phase of mammary gland development occurs mid-gestation, and is marked by the development of epithelial disc-like placodes (9,11). These placodes develop from multilayered ectoderm “milk lines” that arise from proliferation of basal cells in the epidermis, stimulated by signals from the underlying mesenchyme, such as bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 10 (FGF10) (9,11). Overtime, the placodes develop into mammary buds, via elongation and invagination to form a luminal space (10-12). The mammary buds then undergo phases of proliferation and expansion, and give rise to a rudimentary gland (9).

Postnatally, mammary development remains quiescent until puberty (9). During puberty, secondary branching morphogenesis of the ducts occurs in response to hormonal signals, such as estrogen, from the hypothalamic-ovarian-pituitary axis (9,11). These hormonal signals cause the ends of the early ductal structures to transform into terminal end buds (9). The terminal end buds elongate and branch to eventually form full ductal networks throughout the entire
mammary fat pad, a specialized stromal compartment composed of multiple cell types including fibroblasts, adipocytes, endothelial cells, and immune cells (10).

The primary role of the mammary gland during adult life is to accommodate pregnancy by responding to hormones that regulate lactation. The mature breast ducts are composed of two layers of cells that radiate from the nipple: an outer layer of basal myoepithelial cells, and an inner layer of luminal epithelial cells (Figure 1.1) (10). The myoepithelial cells have multiple responsibilities in the breast duct. First, they are responsible for production of basement membrane, which anchors the duct to the extracellular matrix (ECM), and separates epithelial and stromal compartments within the mammary fat pad (12). Second, the myoepithelial cells are involved in mediating differentiation, polarity and reorganization events of the luminal epithelial cells (12). For example, studies by Bissell, Peterson and colleagues have shown that in vitro culture of normal human luminal epithelial cells on Matrigel, a reconstituted source of basement membrane derived from Englebreth-Holm-Swarm sarcomas in mice, supports normal expression of polarity markers including epithelial specific antigen (ESA) and MUC1 (which demarcate the apical membrane), and integrin $\beta 4$ (which demarcates the basolateral membrane) (13). In contrast, culture on Collagen Type 1 does not support this differentiation process (13). Interestingly, introduction of a myoepithelial co-culture restores epithelial polarity even in the absence of Matrigel (13), demonstrating the importance of this cell type in maintaining normal duct structure.
Figure 1.1 Human breast anatomy. The lobes of the breast are connected to the nipple through the ducts, supported by a surrounding stroma. The mature breast ducts are composed of two layers of cells that radiate from the nipple: an outer layer of basal myoepithelial cells, and an inner layer of luminal epithelial cells. The myoepithelial cells are responsible for basement membrane production, and organization of the luminal epithelial cells, which produce milk.

The luminal epithelial cells of the breast duct are responsible for milk production (10). One key hallmark of mammary gland development during pregnancy is proliferation and differentiation of the terminal end buds in response to hormone signals (predominantly progesterone and prolactin) to form secretory milk-producing alveoli (14,15). These alveoli are organized into structures called lobules. Following lactation, the alveoli undergo involution and apoptosis to remove 80% of the epithelium (9). This cycle continues in women with each pregnancy.

1.2 The acquisition and progression of breast cancer

Given that mammary epithelial cells are capable of continual and repetitive differentiation and remodelling events in response to various microenvironmental signals, it is not surprising that they are likewise receptive to signals that may contribute to the generation of cancer. Breast cancer arises from the epithelial cells of the ducts or the lobes of the breast, called ductal carcinoma and lobular carcinoma, respectively. The ducts are the most common origin of breast cancer and will be of primary focus here (Figure 1.2).

In response to genetic or epigenetic alterations within cells, epithelial cells display intraductal proliferation of three non-invasive varieties, including usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), and ductal carcinoma in situ (DCIS) (16). Both UDH and ADH are considered “pre-cancerous”, and there is conflicting evidence about whether they necessitate progression to cancer (16,17). DCIS is the first official stage of breast cancer,
Normal Duct
Usual Ductal Hyperplasia (UDH)
Atypical Ductal Hyperplasia (ADH)
Ductal Carcinoma *In Situ* (DCIS)
DCIS with microinvasion
Invasive Ductal Carcinoma (IDC)
Figure 1.2 Breast cancer progression. Breast cancer starts in the lobe or the duct of the breast. During ductal carcinoma progression, cells become hyperplastic and/or atypical, and eventually progress to ductal carcinoma in situ (DCIS). DCIS is characterized by an intact basement membrane and layer of myoepithelial cells. During invasive ductal carcinoma (IDC), the basement membrane and myoepithelial cell layer is breached, as tumour cells undergo metastatic dissemination.

Adapted from website: http://ftsask.ca/Christine/BC_Types.html
and is characterized by an intact basement membrane and myoepithelial cell layer, and occupation of the intraluminal space by tumour cells (16). DCIS are relatively easy to treat with excision and radiation therapy; however, they are difficult to diagnose and are usually only found using mammography (18).

DCIS lesions are classified into low-, medium-, or high-grade largely based on histological criteria. Low-grade DCIS is characterised by monomorphic cells that are evenly spaced, with small central nuclei. They have few mitoses and do not often display necrosis. In contrast, high-grade DCIS is characterised by pleomorphic cells that are unevenly spaced, with large nuclei. They have many mitoses and often display necrosis. Medium-grade DCIS are more difficult to define as they display features in between low- and high-grade, for example, mild to moderate pleomorphism (16).

Invasive ductal carcinoma (IDC) is characterised by tumour invasion through the basement membrane, the myoepithelial cell layer, and the extracellular matrix (ECM). The transition from DCIS to IDC is typified by a drastic phenotypic transformation, whereby epithelial cells lose many hallmark features that define them as “epithelial”, and adopt a poorly-differentiated phenotype. For example, loss of epithelial (E)-Cadherin, a key cell-cell adhesion molecule expressed on epithelial cells, is associated with the transition from DCIS to IDC, and also with high histological grade, negative estrogen receptor status, and shorter disease-free-survival (19). In patients, acquisition of IDC is associated with an elevated risk of metastasis, even in lymph node-negative
lesions (20). Understanding the mechanisms that mediate this pivotal transition during disease progression is a central focus of cancer research worldwide.

Interestingly, it has been postulated that breast cancer progression may not follow a linear disease course from DCIS to IDC. Rather, studies have described evidence for non-linear mechanisms of cancer progression. This concept is based largely on observations that (a) abnormal molecular characteristics in IDC are often present in DCIS (6,21,22), and (b) tumours are heterogeneous, whereby independent clones can co-exist and progress differently within the same lesion (reviewed in (23)). In order to characterize how breast cancer progresses from DCIS to IDC, one study generated mathematical algorithms to evaluate which of several candidate models of breast cancer progression most accurately recapitulated clinical observations (24). Results indicated that models describing a parallel evolution of cancer (i.e. DCIS and IDC lesions progress in parallel from a common progenitor) mirrored clinical observations better than models describing a linear-type progression (i.e. DCIS is a progenitor of IDC) (24). Their results explain why some breast cancers display features of both DCIS and IDC, and suggest that these types of cancers may arise from a common progenitor rather than by linear evolution, adding another layer of complexity to understanding breast cancer progression.

1.3 Breast cancer classifications

Breast cancer is classified based on stage (25) and grade (26). The TNM classification system of breast cancer stage is based on the size of the tumour
(T), lymph node involvement (N), and the presence/absence of distant metastases (M) (27,28). Generally, stage 1 includes small tumours (<2 cm) with or without micrometastasis involvement in the axillary lymph nodes (under the arm). Stage 2 tumours are slightly larger, (2-5 cm), with or without micrometastasis involvement in the axillary lymph nodes. Stage 3 tumours range in size, and are more locally advanced in that they have lymph node involvement under the arm, near the breastbone, or around the collarbone, and/or have spread to the chest wall, or the skin. Stage 3 tumours are sometimes inoperable by radical mastectomy, especially in cases of inflammatory breast cancer, in which lymph and skin metastases are significant. Stage 4 tumours show signs of metastases in other organs, most often including lung, liver or bone, and require treatment regimens that will simply maintain disease (27,28).

While tumour stage is a measure of size and extent, tumour grade is a measure of histological features. Histological grade is based on three main criteria, including tubular formation, nuclear atypia, and mitotic counts, of which nuclear atypia has been reported to be the best predictor of recurrence following treatment (29-31). Each criterion is independently graded: Grading of tubular formation is based on whether the tumour is papillary, tubular or cribriform (grade 1), or exhibits a solid nest pattern (grade 3). Grading of nuclear atypia is based on whether the tumour cells have nuclei that are uniform (grade 1) or pleomorphic (grade 3). (Note that grade 2 for both tubular formation and nuclear atypia is “in between” respective grade 1 and grade 3 features.) Finally, grading of mitosis is based on whether there are less than 5 mitoses per 10 high-power
fields of view (grade 1), between 5-10 mitoses (grade 2), or more than 10 mitoses (grade 3). After each criterion is graded, a final tumour grade is assigned based on the sum of all grades (Tumour Grade 1= sum 3-4; Grade 2= 5-7, Grade 3= 8-9), where grade 1 is well-differentiated and associated with good prognosis, and grade 3 is poorly-differentiated and associated with poor prognosis (29-31).

In addition to traditional histological and clinical classifications of breast cancer, DNA microarray analyses have uncovered several molecular subtypes of breast cancer that have helped predict patient-specific survival, and contributed to the development of targeted therapies. In a widely influential article published in *Nature*, Perou *et al.* defined four molecular subtypes of human breast cancer based on gene expression patterns in 42 different human breast tumour samples, using cDNA microarrays reflecting 8,102 human genes (6). From this study, four molecular subtypes of breast cancer were defined, including normal breast-like, ER+/luminal-like, ErbB2+, and basal-like (6). **Normal-like** tumours exhibit high expression of epithelial and adipocyte markers, and low expression of luminal epithelial markers. **ER+/luminal-like** tumours are estrogen receptor (ER) positive (+) and overexpress luminal cell markers. Recent studies have reported that the ER+/luminal-like subtype should be split into Luminal A (which is the most common subtype) and Luminal B (7,8), based on low and high histological grade, respectively. Patients bearing luminal-like tumours tend to have a good prognosis compared to other molecular subtypes. **ErbB2+** (also known as HER2+) tumours overexpress the *ERBB2* oncogene and are progesterone receptor (PR) negative (-) and ER-. **Basal-like** tumours (also
known as Triple Negative) are PR-/ER-/HER2-, and exhibit high gene expression of Keratins 5, 6, and 17 (6). Interestingly, studies have revealed that carriers of the \textit{BRCA1} mutation, or the \textit{TP53} mutation, are predisposed to the basal-like subtype (7,8). Furthermore, basal-like and ErbB2+ subtypes are associated with shortest overall patient survival, and shortest relapse-free survival (7). More recently, another subtype has been identified as \textbf{Claudin-low}. This subtype is often triple negative (PR-/ER-/HER2-) and has low expression of cell-cell adhesion proteins, such as E-cadherin (32,33), making it an aggressive subtype of breast cancer.

\textbf{1.4 The metastatic cascade}

\textit{IDC} is a trigger and initiation step for the onset of the metastatic cascade. Metastasis is the process by which cancer cells spread and establish a secondary tumour at a distant organ site. Before metastasis occurs, breast cancer is generally considered to be “curable” by successful surgical resection and chemotherapy (16,18). However, once cancer has spread beyond the breast and lymph nodes, treatment modalities are usually geared towards disease maintenance and prolonging survival. Given the association between metastasis and poor prognosis, understanding the metastatic cascade poses an intriguing and challenging problem for scientists.

In breast cancer, metastasis to lung, liver and bone are the most common causes of death (34,35). During metastasis, primary tumour cells must acquire the capacity to invade the basement membrane and myoepithelial cell layer
surrounding the breast duct. They proceed to degrade and invade through the ECM, and recruit a vasculature. Once metastatic cancer cells reach the vascular network, they intravasate into circulation, migrate through the bloodstream, and extravasate into secondary tissue sites. Alternatively, the cancer cells may spread through the lymphatics. At the secondary site, metastatic cancer cells must be able to sustain their growth in order to successfully form a secondary tumour mass (Figure 1.3) (2,36-38).

The metastatic process is extremely delicate and inefficient, and some steps are “easier” to complete than others (36). As eloquently described by Hanahan and Weinberg in *The hallmarks of cancer* (39), and more recently in *Hallmarks of cancer: The next generation* (3), there are several cellular functions that are essential for successful metastasis, and not every tumour cell is capable of all of these functions. Examples include enhanced proliferation, evasion of apoptosis, tumour angiogenesis, and cellular invasion, which will constitute the main chapters of this thesis.

1.4.1 Enhanced proliferation and evasion of apoptosis

Under normal physiological conditions, cell fate is determined by incoming signals from the microenvironment. These signals bind to cell surface receptors to initiate a cascade of intracellular communication events that ultimately end in a change in gene expression and, subsequently, cellular behaviour. Two fundamental behavioural hallmarks of cancer include enhanced proliferation and evasion of apoptosis (3,39). Indeed, while most normal cells will not assume a
Invasion

Vascularization

Intravasation into blood vessels

Migration through the blood stream

Extravasation at secondary site

Establishment of metastases
Figure 1.3 The metastatic cascade. During breast cancer metastasis, primary tumour cells must acquire the capacity to invade the basement membrane and myoepithelial cell layer surrounding the breast duct. They proceed to degrade and invade through the extracellular matrix, and recruit a vasculature. Once metastatic cancer cells reach the vascular network, they intravasate into circulation, migrate through the bloodstream, and extravasate into secondary tissue sites. At the secondary site, metastatic cancer cells must be able to sustain their growth in order to successfully form a secondary tumour mass. Metastasis to lung, liver and bone are the most common causes of death.

proliferative phase unless instructed to do so, cancer cells tend to be self-sufficient in their ability to sustain mitogenic signalling. Furthermore, cancer cells can harbour mutations that render them insensitive to normal apoptotic signals from their microenvironments. Not surprisingly, mutations in genes associated with the cell cycle or with apoptosis are frequent in cancer. An understanding of these hallmarks and how their cycles are regulated is crucial to preventing progression of disease.

1.4.1.1 The cell cycle

The mammalian cell cycle consists of four phases that accommodate for DNA replication and cellular division, including G₁, S, G₂ (together known as interphase), and mitosis (Figure 1.4). G₁ (“first gap”) is the first phase after a cell division. RNA and protein are produced immediately after the emergence of daughter cells, however, DNA synthesis rests, while cells decide whether to continue to divide, become quiescent or differentiate. At this point, cells can enter a quiescent phase, G₀, before proceeding with the cell cycle. The decision to maintain G₀ is often governed by an absence of mitogenic signals, a loss of adhesion, or cell density contact-inhibition (40). Entrance into G₀ is usually reversible, as cells re-enter the cell cycle in the presence of mitogenic signals (40). However, entrance into G₀ can also be irreversible, in which case cells are said to be “post-mitotic” (41). For example, terminally differentiated neurons of the central nervous system are post-mitotic, and re-entrance back into the cell cycle upon de-regulation of this post-mitotic state is associated with the
Figure 1.4 The cell cycle. The mammalian cell cycle consists of four phases that accommodate for DNA replication and cellular division, including \( G_1 \), \( S \), \( G_2 \) (together known as *interphase* (I)), and mitosis (M). Progression through the cell cycle is regulated by protein complexes (ex. cyclin and cyclin-dependent kinase complexes) or epigenetic alterations (ex. phosphorylation of Histone H3) that cycle with phase-entry and -exit events.

*Adapted from website:*
acquisition of neurodegenerative diseases such as Alzheimer’s Disease (41,42). Re-entrance into the cell cycle from \(G_0\) or \(G_1\) permits entry into S (“synthesis”) phase during which DNA is copied. Following S phase is \(G_2\) (“second gap”), which is the final rest phase before **mitosis** (43).

Mitosis is the process of cell division, and consists of four phases, prophase, metaphase, anaphase and telophase. During **prophase**, the loosely bundled chromatin condenses to form chromosomes. Centrosomes, composed of two centrioles, move apart around the nuclear envelop in preparation for microtubule organization (44,45). During **metaphase**, the chromosomes line up along the equatorial plane, and when the nuclear envelop breaks down, microtubules from the polar centrosomes “search-and-capture” kinetochores on sister chromatids (44). During **anaphase**, the proteins that link sister chromatids are cleaved, and microtubules begin to shorten and cause segregation of sister chromatids towards opposite poles of the cell (43). Abnormal segregation of sister chromatids can cause irreversible damage to cells, therefore tight checkpoints exist to ensure normal segregation (43). During **telophase**, a nuclear membrane assembles around daughter chromosomes, and chromosomes decondense back into chromatin. Following mitosis, **cytokinesis** occurs, whereby a contractile ring, called a *cleavage furrow*, develops at the equatorial plane to finally split the cytoplasm to make two daughter cells (46). The daughter cells proceed to exit mitosis and enter back into \(G_1\) in response to anaphase-promoting complex/cyclosome (APC/C) assembly (43), and repeat the cell cycle to sustain proliferation.
The cell cycle is regulated by several mechanisms. First, cyclins and cyclin-dependent kinases (CDKs) mediate many of the phase-entry and -exit events during the cell cycle. For example, transition from $G_1$ to $S$ phase involves activation of the cyclinD-CDK4/6 complex (47), and transition from $G_2$ to mitosis involves activation of the cyclinB-CDK1 complex (Figure 1.4) (48,49). Second, the cell cycle has several “checkpoints” that ensure each step is completed before proceeding to the next step. For example, during metaphase, in order to ensure proper segregation of sister chromatids, the spindle assembly checkpoint ensures that all chromosomes are lined up at the equatorial plane and bound to the mitotic spindle before anaphase can occur (44). The cell cycle is also regulated epigenetically. For example, phosphorylation of histone H3 fluctuates at various stages of mitosis, and is most prevalent during metaphase (Figure 1.4) (50). Furthermore, phosphorylation of histone H3 drops abruptly upon mitotic exit, and thus serves as a good marker for mitogenic activity (50). Together, these regulatory mechanisms are critical to ensure proper cellular division.

1.4.1.2 Cancer cells exhibit self-sufficient proliferative programmes

There are several mechanisms that cells hijack to autoregulate their own proliferative programmes, rather than relying on regulated microenvironmental cues. First, cancer cells can aberrantly produce and secrete mitogenic signals themselves that signal back in an autocrine fashion. One example of this phenomenon is the production and secretion of WNT proteins from breast cancer cells (51). WNT proteins are a highly conserved family of secreted glycoproteins
that are heavily studied in developmental biology. Mutations in WNT pathway members are common in various types of cancer, most notably colorectal and skin cancers (52,53), to induce constitutive and aberrant WNT signalling; however, these mutations are not as common in breast cancer. Rather, breast cancer exhibits constitutive autocrine mitogenic WNT signalling, whereby WNT signals are upregulated via inhibition of sFRP1 expression, a secreted factor that competes with WNT ligands for receptor-binding (51,54,55). Interestingly, autocrine WNT signals in breast cancer are not only capable of activating canonical WNT signalling, but also activate ERK1/2 through transactivation of epidermal growth factor receptor (EGFR; also known as HER1) to mediate its mitogenic effects (51).

Another example of how cells regulate their own proliferative programs is through altered receptor expression to improve receptivity to growth signals. One example is human EGFR expression during cancer progression. Similar to HER2 (which defines an aggressive molecular subtype of breast cancer as described in section 1.2.1), EGFR is part of the ErbB-family of receptor tyrosine kinases, and its overexpression has been linked to various types of cancer, including breast, ovarian, stomach, and uterine cancers (56). Upon ligand activation, EGFR can stimulate a plethora of signalling cascades that are involved in activating proliferative programmes, including ERK MAPKs, PI3K/Akt, and STAT (57,58). Overexpression of ErbB-family receptors can also cause ligand-independent receptor activation to propagate these mitogenic pathways (39,58).
Lastly, cells can autoregulate their proliferation through mutations in intracellular signalling components that support proliferative programmes. For example, mutations in the small GTPase RAS result in enhanced proliferation in many types of human cancers including colon, bladder, and lung, among others (59-62). Furthermore, several studies by Clevers, Vogelstein, Kinzler and colleagues reported that mutations in β-catenin or APC (which mediate WNT signalling) play a significant role in the acquisition of colon cancer (52,63). Mutational alterations in these pathway components lead to constitutive, unregulated signalling propagation, and ultimately, the acquisition of disease.

1.4.1.3 Programmed Cell Death

Apoptosis, or programmed cell death, is a highly regulated and conserved mechanism amongst multicellular organisms that ensures tissue homeostasis. In contrast to necrosis, which results from cell trauma or injury, apoptosis is vital to an organism’s normal development and sustainability. Apoptosis is most often mediated by executioner proteins called caspases, a group of cysteine proteases that contain a pro-domain in their latent form. Activation of caspases is mediated by several mechanisms, including, but not limited to, (i) inhibition of caspase-inhibitors, (ii) assembly of the apoptosome, or (iii) death receptor activation (Figure 1.5). There are two types of caspases, initiator caspases (ex. caspases 8, 9, and 10) and effector caspases (ex. caspases 3, 6, and 7). In general, caspase-8 is the main initiator for cell death receptor-induced apoptosis, while caspase-9 mediates mitochondria-induced apoptosis (39). Upon activation,
**Figure 1.5 Mechanisms of Apoptosis.** Apoptosis is mainly mediated by one of three mechanisms: (i) Inhibition of caspase inhibitors (such as Inhibitor of Apoptosis Protein, or IAP), which allows for activation of latent caspases, including pro-caspase-9, in response to mitochondria-secreted Small Mitochondria-derived Activator of Caspases (SMAC). (ii) Apoptosome assembly, which is initiated by the release of cytochrome c from the inner membrane of the mitochondria, via formation of the Mitochondrial Apoptosis-induced Channel (MAC). MAC formation is normally regulated by an interplay between Bcl-2 (which inhibits channel formation), and Bax (which promotes channel formation). (iii) Death Receptor activation, which initiates apoptosis through mitochondria-independent signal transduction and/or caspase activation. In all cases, activation of caspases results in degradation of cellular components and organelles to cause death.
initiator caspases cleave and activate effector caspases, which proceed to cleave organelles or proteins within the cell to induce apoptosis, such as Poly-ADP-ribose polymerase-1 (PARP-1) (64). Of note, PARP-1 can also induce apoptosis in the absence of caspases, by mediating translocation of Apoptosis Inducing Factor (AIF) from the mitochondria to the nucleus (64).

Activation of caspases often occurs through mitochondrial release of pro-apoptotic proteins that enable caspase activity. For example, in response to nitric oxide (NO), the mitochondrial membrane becomes more permeable to pro-apoptotic proteins that are secreted into the cytosol (65). One such mitochondria-derived protein is Second Mitochondria-derived Activator of Caspases (SMAC). SMAC binds to Inhibitor of Apoptosis Protein (IAP) to prevent it from inhibiting caspase-9 activity, thereby activating apoptosis (66). Indeed, addition of SMAC mimetics to cell lines (including breast, colon, lung, pancreas, skin and prostate cancer cell lines) increases sensitization to chemotherapy-induced apoptosis (67).

Another mechanism of mitochondrial-induced apoptosis is through formation of the apoptosome complex. The apoptosome is initiated by the release of cytochrome c from the inner membrane of the mitochondria, via formation of the Mitochondrial Apoptosis-induced Channel (MAC) (68). MAC formation is normally regulated by an interplay between Bcl-2 (which inhibits channel formation), and Bax (which promotes channel formation) (68-70). Once MAC has been formed, cytochrome c is released from the inner membrane of the mitochondria, and forms the apoptosome by binding to Apoptotic Protease
Activating Factor-1 (APAF1) and pro-caspase-9 (68). The caspase pro-domain is subsequently cleaved off, and caspase-9 becomes activated to induce apoptosis.

In addition to mitochondria-mediated apoptosis, signal transduction in response to ligand binding to death receptors can initiate apoptosis through mitochondria-independent caspase activation. For example, interactions between members of the Tumour Necrosis Factor (TNF) receptor family and their ligands induce apoptosis through activation of the TNF receptor-associated death domain (TRADD), and subsequent activation of caspase-8 (71). Indeed, treatment of cell lines (ex. Hodgkin’s lymphoma and breast cancer cell lines) with TNF-α or related ligands has been shown to induce sensitization to chemotherapy-induced apoptosis (72,73).

1.4.1.4 Alterations associated with apoptosis evasion

In cancer, apoptotic programmes are often deregulated to render the cell unreceptive to initiation of cell death pathways. Like proliferation, apoptotic programmes can be altered on several levels, including altered mitochondrial function, or mutations in apoptotic signalling pathways. For example, mitochondria-induced apoptosis can be deregulated in cancer through altered expression of Bcl-2 or Bax proteins. Specifically, in breast cancer cells, the pro-tumourigenic progesterone metabolite 5-α-dihydroprogesterone causes an increase in the ratio of Bcl-2:Bax mRNA expression concomitant with decreased apoptosis, while the anti-tumourigenic progesterone metabolite 3-α-dihydroprogesterone has the opposite effect (74).
Mutations in genes associated with apoptosis are additional mechanisms of apoptosis evasion in cancer. One example that has received great attention is the tumour suppressor p53 (encoded by the \textit{TP53} gene). The normal function of p53 is to prevent damaged cells from replicating. Therefore, mutated p53 permits replication of cells with DNA damage, which can lead to an accumulation of genetic mutations and the initiation of cancer (39,75,76). Another example of a common mutation in cancer is deactivation of the \textit{BCL2} oncogene, which renders cells unable to initiate the apoptosome (39,68,69).

\subsection*{1.4.2 Tumour vascularization}

As tumours proliferate, they eventually require a larger supply of oxygen and resources to sustain their growth. Accordingly, tumour vascularization is a rate-limiting step in the metastatic cascade. In addition to supporting growth, tumour-associated vessels facilitate cancer progression by acting as conduits for tumour cells to travel to secondary sites. Tumour vascularization involves cooperation between multiple cell types within the local microenvironment, and recruitment of bone marrow-derived cells (BMDCs) from distant sites through chemokine signalling axes (77). Together, these cell types work in concert within a pro-angiogenic niche that is conducive to vascular development, to “feed” the growing tumour and promote disease progression. Of note, high microvascular density is associated with poor prognosis in cancer patients (78,79), and is therefore an interesting target for therapy.
1.4.2.1 Structure of blood vessels and the importance of vessel integrity

Blood vessels are made of multiple cell types, including an external layer of pericytes, a basement membrane, and an internal layer of endothelial cells (Figure 1.6) (80). Endothelial cells are the main components of the vessel walls and are tightly assembled to ensure vessel integrity, while pericytes play a role in vessel maturity (80). Blood vessels can become “leaky” during cancer progression, largely due to destabilization of the vessel structure (77,80). For example, during hypoxia, there is an upregulation of vascular endothelial growth factor (VEGF) that causes endothelial cells to exhibit looser cell-cell adhesions (81). Furthermore, hypoxia causes an upregulation of Angiopoietin-2, which is associated with a depletion of pericyte coverage, reduced basement membrane integrity, and loosening of the endothelial cell layer (77,82). Lastly, hypoxia inducible factor (HIF) proteins have been shown to recruit BMDCs that secrete proteases that degrade extracellular matrix and basement membrane components, such as matrix metalloproteinases (MMPs) and cathepsins (83). Together, deregulation of vessel integrity supports elevated intra/extravasation of tumour cells across the vessel wall, and reduces drug delivery to the tumour site. As a result, “vessel normalization” has been proposed as a potential mechanism of improving drug delivery during treatment, involving increasing pericyte coverage, basement membrane deposition, and tightening of endothelial cells (80,84).
Pericytes

Basement membrane

Endothelial cells
Blood vessels are made of multiple cell types, including an external layer of pericytes, a basement membrane, and an internal layer of endothelial cells. Endothelial cells are the main components of the vessel walls and are tightly assembled to ensure vessel integrity, while pericytes play a role in vessel maturity. Blood vessels can become leaky during cancer progression, due to destabilization of the vessel structure.

*Adapted from Bergers and Benjamin (2003) Nat Rev Cancer, 3:401-410*
1.4.2.2 Mechanisms of tumour vascularization

Tumour vascularization occurs through a combination of vasculogenesis (the de novo growth of blood vessels), vasculogenic mimicry (cancer cells mimic endothelial cells and make their own vessels) and angiogenesis (the sprouting of new vessels from existing vessels) (85-87). Although each of these processes will be discussed, angiogenesis is the primary mechanism of tumour vascularization, and will be of primary focus.

In adult systems, vasculogenesis is mediated by mobilization of BMDCs, which contain a variety of progenitor populations that give rise to cells that constitute the blood, vasculature, supportive tissue and bone. BMDC populations are mobilized into the blood stream in response to chemokine signals, and home to tissues that require stem cell populations for tissue regeneration, repair, and vascularization. Likewise, studies by Rafii, Lyden, Kaplan and colleagues have demonstrated that BMDCs are recruited to both pre-metastatic and primary tumour sites in response to factors secreted by tumour cells to aid in vascularization (88,89). In one such study, they report that bone marrow-derived endothelial and hematopoietic cell types are required for tumour vascularization in vivo (90).

Vasculogenic mimicry is another mechanism that yields de novo vessels that contribute to tumour vascularization (86,87). Vasculogenic mimicry is characterized by the ability of aggressive cancer cells to express endothelial cell markers and form functional endothelial-like vascular networks (86,91). Hendrix and colleagues have shown evidence for vasculogenic mimicry in melanoma,
ovarian and prostate cancer (92,93), and other groups have demonstrated the same phenomenon in lung and breast cancer (94,95). Vasculogenic mimicry will be discussed in more detail, as an example of how tumour cells display plasticity during disease progression (Section 1.5.1).

Tumour angiogenesis, on the other hand, is a more widely studied phenomenon that involves sprouting of vessels from pre-existing networks (c.f. de novo vessel formation). In fact, as first proposed by Judah Folkman over 40 years ago in the New England Journal of Medicine (96), many current cancer therapies target mediators of tumour angiogenesis, such as VEGF (Avastin/Bevacizumab). During normal tissue repair, such as during tissue ischemia, angiogenesis is initiated in response to low oxygen (hypoxia) (97,98). Subsequently, HIF proteins induce the upregulation of pro-angiogenic proteins, such as VEGF, FGF, or platelet-derived growth factor (PDGF), in order to induce vessel branching and oxygen delivery (97,98). Angiogenesis is similarly initiated in cancer when tumours become large enough that oxygen cannot permeate all the way through (approximately 1-2 mm (87)). The resulting hypoxia gradient ignites a signalling cascade that induces the expression of pro-angiogenic proteins (99).

1.4.3 Cellular invasion

During cancer progression, epithelial organization is disrupted when cancer cells become invasive. Cellular invasion is not a single process, but rather a composite of multiple cellular functions and alterations that work together in
synchrony. One pivotal alteration that occurs during the acquisition of an invasive phenotype is **epithelial-to-mesenchymal transition (EMT)** (3,100). EMT is a key aspect of tissue remodelling in normal physiological processes including gastrulation, lactational involution, and wound healing (100). Equally, EMT contributes to tissue remodelling during cancer invasion, by mediating changes in cell-cell adhesions, including downregulation of E-Cadherin and upregulation of N-Cadherin (101,102). These alterations allow cancer cells to break free of their site-of-origin.

A second process involved in cellular invasion is proteolytic degradation of extracellular matrices. Similar to EMT, **proteolytic degradation** is not limited to cancer invasion, but rather is a common characteristic of tissue remodelling. For example, matrix metalloproteinases (MMPs) are a family of proteases that are essential during tissue remodelling in both normal and pathological contexts. During mammary gland development, MMP activity is important in supporting the invasive events that are required for branching and alveolar morphogenesis (103-106). In cancer, MMPs have been shown to promote invasive phenotypes. For example, MMP activity is upregulated by insulin-like growth factor-1 (IGF-1) in human breast cancer cell lines, through MAPK and Akt pathways leading to increased invasive potential (107).

Once cell-cell adhesions have been disrupted and the surrounding ECM has been sufficiently degraded, cancer cells are free to migrate away from their site-of-origin. It has been proposed that **cellular migration** is mediated by guidance interactions between multiple cell types in the tumour milieu. For
example, Condeelis and colleagues have shown that tumour cell migration through the ECM and into the blood stream (intravasation) is mediated by a paracrine interaction between tumour cells and infiltrating macrophages, involving a protein called MENA that is expressed on tumour cells (108). Using intravital multiphoton imaging, this group was able to observe interactions between alternating macrophages and migratory cancer cells \textit{in vivo} in real time (109), illustrating the importance of stromal compartments in supporting migratory phenotypes in cancer.

1.5 \textit{Cellular plasticity as a mechanism for metastasis}

\textit{(See copyright release Appendix 1)}

Given the inefficiency of the metastatic cascade, it is believed that tumour cells that successfully metastasize display phenotypic plasticity. Studies in the field of induced pluripotent stem cell (iPSC) biology support this concept, as alterations which support tumourigenesis also promote the induction of pluripotency (110-112). In cancer, cellular plasticity is characterized by a loss of lineage-specific markers and an ability to mimic embryonic cell types, yielding cells that are highly resilient and self-sufficient, and that can survive in multiple dynamic foreign microenvironments throughout the metastatic cascade (113). A few examples of how cells display plasticity during cancer progression include vasculogenic mimicry, EMT, endothelial-to-mesenchymal transition (EndMT), and phenotypic switching:
1.5.1 Vasculogenic mimicry

Several studies have demonstrated the importance of cellular plasticity in cancer. A clear example is vasculogenic mimicry, characterized by the ability of aggressive cancer cells to express endothelial cell markers and contribute to the formation of de novo endothelial-like vascular networks (86,91). These networks develop via a process similar to normal embryonic vasculogenesis, and are thought to be functional conduits for circulation throughout the tumour (86). One mediator of vaculogenic mimicry in cancer is VE-Cadherin. Notch4 and Nodal have been shown to cooperate to induce VE-Cadherin expression and the transendothelial phenotype in aggressive melanoma cells (114). Indeed, knockdown of VE-Cadherin, Notch4, or Nodal in melanoma cells impairs the formation of vasculogenic networks (114-117). Vasculogenic mimicry has been observed in melanoma, breast cancer, prostate cancer, ovarian cancer and lung cancer, and exemplifies the ability of cancer cells to alter their gene expression to resemble cell types that are not part of their own lineage (92-95).

1.5.2 Epithelial-to-mesenchymal transition (EMT)

EMT is another example of plasticity, and can be seen both during normal development and in cancer (118). EMT is characterised by a loss of epithelial cell markers, such as E-Cadherin, and the acquisition of mesenchymal markers, such as Vimentin and N-Cadherin (118). During development, EMT is required for gastrulation and serves an essential morphogenic function (119). In mammalian embryogenesis, the primitive streak is the site of involution during gastrulation,
mediated by signals from the node including Nodal and FGF (119). Signals from the node induce the expression of transcription factors that are essential for EMT, including Snail and Twist, which in turn alter expression of E-Cadherin both at the gene and protein level to facilitate motility (119). In cancer, EMT is likewise correlated with a broad upregulation of pluripotency markers, including Snail and Twist transcription factors (118-121). Functionally, EMT is associated with increased migration and invasion, changes in cell adhesion, intravasation of cancer cells into the blood stream, and spontaneous metastasis, all attributable to robust changes to cell-cell adhesion molecules during this transition (118).

1.5.3 Endothelial-to-mesenchymal transition (EndMT)

Another interesting example of cellular plasticity similar to EMT is EndMT, a transdifferentiation event (122). EndMT is characterized by a loss of endothelial cell markers such as vascular endothelial (VE)-Cadherin and CD31, and the acquisition of mesenchymal markers such as SNAI1 to yield an invasive cellular phenotype (122). EndMT occurs in normal embryogenesis, such as during heart valve development, whereby myocardial-derived TGF-β induces SNAI1 expression and subsequent downregulation of VE-Cadherin in endocardial endothelial cells of the heart tube (123). This EndMT event allows endothelial cells to delaminate, activate proteolytic enzymes and invade the ECM to initiate valve formation (123). Similarly, in response to tumourigenic signals within the tumour microenvironment, EndMT is believed to cause delamination of endothelial cells from local blood vessels. These endothelial cells are thought to
contribute to the population of cancer-associated fibroblasts (CAFs) that are present in the tumour stroma, which are a major source of fibronectin, secreted oncoproteins and growth factors (122,124).

1.5.4 Phenotypic “switching”

Several interesting studies have shown that sorted tumour cell subpopulations exhibit phenotypic switching, and display dynamic cell surface marker expression. Meyer et al. found that non-invasive epithelial-like CD44+/CD24+ breast cancer populations gave rise to highly-invasive mesenchymal-like CD44+/CD24- cells both in vitro and in vivo (125). Interestingly, the phenotypic switching events that occurred in each population were dependent on Activin/Nodal signalling (125). Consistently, Morrison and colleagues showed that melanomas sorted for ABCB5+ versus ABCB5- were able to re-establish cell surface marker expression ratios that recapitulated the original tumour population in vivo (126). This switching phenomenon was also true for many other markers, including CD166, A2B5, CD151, CD54, CD44, CD9, CD29, N-Cadherin, and CD271. Phenotypic switching is another emerging example of how tumours cells display plasticity.

1.6 Embryonic proteins promote plasticity in cancer

One commonality between the aforementioned examples of cellular plasticity is that these processes frequently involve embryonic programmes. Recently, there has been a surge of research dedicated toward identification of
embryonic factors that are aberrantly expressed in cancer, and responsible for promoting cellular plasticity during cancer progression. Hendrix and colleagues elegantly tackled this problem by using the zebrafish embryo as a functional in vivo reporter to gain insight about what embryonic pathways might be activated in cancer cells (127). In their study, aggressive melanoma cells (versus poorly aggressive melanoma cells) were injected into the animal pole of zebrafish embryos at the blastula stage, and the effect of the melanoma cells on host development was observed after 6-8 hours. Interestingly, the aggressive melanoma cells were able to induce an almost-complete secondary axis, but the poorly aggressive melanoma cells were not. The pressing question was, what embryonic factor is able to induce a secondary axis?

Several studies implicated the embryonic protein Nodal as a top candidate for the observed phenomenon (128,129). It had been previously shown that injection of Nodal RNA into zebrafish embryos could induce a secondary notochord (128). Furthermore, an article had been published in Nature demonstrating that misexpression of Nodal homologue, Znr1, could induce an ectopic outgrowth in zebrafish embryos (129). Together, these findings were similar to those observed in response to aggressive melanoma cell injections (by the Hendix group), and therefore pointed to Nodal as a fundamental embryonic protein with a putative role in cancer progression.
1.6.1 Nodal: An early embryonic protein

(See copyright release Appendix 1)

Nodal is an embryonic morphogen that promotes mesendoderm specification and left-right asymmetry during embryogenesis (127,130,131). This embryonic protein is also essential for maintaining the pluripotent quality of stem cells during development, and is down-regulated as stem cells differentiate (127,130). Nodal is part of the TGF-β superfamily, and its actions are initiated by binding Activin-Like Kinase Receptor type I (ALK4/7) and type II (ActRlIB) (Figure 1.7). Upon activating this receptor complex (ALK receptor complex), SMAD2 (and possibly SMAD3) is phosphorylated intracellularly and interacts with SMAD4 before translocating to the nucleus (130). In the nucleus, transcription factors, such as forkhead box H1 (FOXH1), are activated to increase Nodal expression and create a positive feedback loop (127). In a normal adult cell, Nodal expression and subsequent signalling is thought to be silenced (130).

Nodal signalling is enhanced by the epidermal growth factor-crypto/FRL1/cryptic (EGF-CFC) family co-receptor, Cripto (TDGF1) (127). Cripto has an N-terminal signal peptide, an EGF-like domain which directly interacts with Nodal, a conserved cysteine-rich (CFC) domain which interacts with ALK4, and a hydrophobic C-terminal containing sequence information for a glycosyl-phosphatidylinositol (GPI) anchor (132). The adjacent positioning of the EGF-like domain and the CFC domain helps bring Nodal into proximity with ALK4 to facilitate enhanced binding (132). Cripto is either linked to the cell membrane through its GPI-anchor, or exists as a soluble factor in the extracellular space
In the extracellular environment, Nodal binds the activin-like kinase type I (ALK4/7) and type II (ActRIIB) heterodimeric receptor complex and subsequently triggers the phosphorylation and activation of ALK4/7 by ActRIIB. Intracellularly, ALK4/7 phosphorylates SMAD2/3, which in turn forms a complex with SMAD4. The SMAD2/3-SMAD4 complex translocates to the nucleus where it associates with transcription factors, such as forkhead box H1 (FOXH1), to initiate transcription of target genes. Target genes include Nodal (for autoregulation of its own expression), or Lefty (a Nodal inhibitor). Like other TGF-β superfamily proteins, Nodal is a pro-protein that requires cleavage by convertase enzymes, such as Furin (SPC1) and Pace4 (SPC4), in order to become active. Nodal signalling is enhanced by the epidermal growth factor-coreceptor Cripto (TDGF1), which binds to the ALK4/7-ActRIIB receptor on the cell membrane, and is known to be associated with tumourigenesis. The small molecule inhibitor SB431542 interferes with the Nodal signalling cascade by inhibiting the ALK4/7 subunit of the ALK4/7-ActRIIB heterodimeric receptor complex.
Studies have shown that GPI-linked Cripto is required for its role as a Nodal co-receptor (135,136). Studies in *Xenopus* embryos show that Cripto enhances Nodal signalling through direct interactions with ALK-4 that facilitate nodal-receptor binding, or with ALK-7 to enhance receptivity to nodal activation (137,138).

Nodal signalling is inhibited spatially and temporally during development by inhibitors such as LeftyA/B, and Cerberus-like (CER1) (130,139). These inhibitors are transcribed in response to Nodal signalling, and act as a negative feedback mechanism to control Nodal localization and action in the developing embryo (130). Lefty, in particular, is also regulated by alternate SMAD pathways, WNT, and Oct4 (POU5F1) signalling, and is upregulated during differentiation events (140). It inhibits Nodal signalling through interactions with Nodal and/or Cripto in the cellular microenvironment or at the cell surface (130).

The *Nodal* gene is located on chromosome 10 in humans, and contains 3 exons. In mice, *Nodal* is transcriptionally regulated at 3 different sites: the left side-specific enhancer, (LSE), the asymmetric enhancer (ASE), and the node-specific enhancer (NDE) (141,142). The LSE and ASE, located 4kb upstream of the translation start site and in the first intron, respectively, are involved in autoregulation of Nodal signalling through activation of FOXH1 transcription factor (131,141,142). The NDE, located 10 kb upstream of the gene locus, is Notch-responsive (143,144). Similar enhancer regions exist between mice and humans, suggesting similar mechanisms of transcriptional regulation. As expected, human melanoma cells utilize a positive feedback mechanism that
sustains Nodal signalling, and data suggests that Notch regulates Nodal in this cancer type as well (4,114,127,145). Most of the literature surrounding Nodal regulation and signalling is based on murine, zebrafish, and *Xenopus* model systems; the details of *Nodal* transcriptional regulation in human cancer and development are currently being investigated.

*Nodal* transcription may also be regulated by epigenetic mechanisms, such as methylation. The *Nodal* gene contains a CpG island near the transcription start site, and the methylation of this region has been correlated with gene expression in melanoma cells (145). MicroRNA regulation has also been implicated to play a role in post-transcriptional regulation of Nodal. Specifically, miR-430 is able to inhibit translation of the Nodal agonist, Squint, in zebrafish (146). The role of miRNA in the regulation of human Nodal has not yet been described.

In addition to transcriptional and post-transcriptional mechanisms of regulation, Nodal is also subject to post-translational regulation. Studies in murine embryos have determined that Nodal is a pro-protein that is subject to activation by subtilisin-like proprotein convertase (SPC) enzymes, SPC1 (FURIN) and SPC4 (PCSK6; previously known as PACE4) (147). SPC1 and SPC4 cleave and activate Nodal upon secretion, and are necessary for Nodal signalling and Cripto induction *in vivo* (147). Nodal is also post-translationally modified through glycosylation events. Human Nodal has 2 glycosylation sites within its propeptide, and murine Nodal has 1 glycosylation site within its propeptide. In
murine Nodal, N-glycosylation acts to stabilize the pro-domain, and inhibit proteolytic maturation (148).

Biochemical studies using murine Nodal expression constructs have demonstrated that Nodal is trafficked within the cell through both Cripto-dependent and Cripto-independent mechanisms. Previous studies examining the Cripto-dependent mechanism of Nodal trafficking have shown that GPI-anchored Cripto recruits uncleaved Nodal and soluble convertase enzymes to flotillin lipid rafts on the cell membrane, to facilitate pro-domain processing and endocytosis to the early endosome (133,134,149-152). In the early endosome, Nodal can interact with its ALK receptor complex to propagate its signal, and eventually progress to the lysosome (133). Interactions between ALK4 and dapper-2 (DACT2), a dishevelled-associated protein that interacts with the Nodal pathway at the late endosome marked by RAB7, accelerates trafficking to the lysosome (133).

Cripto-independent mechanisms of trafficking can follow several modalities (133,139,153). For instance, unidentified GPI-anchored receptors can behave like Cripto by recruiting uncleaved Nodal and soluble convertase enzymes into proximity to facilitate cleavage (133). It is also possible that uncleaved Nodal interacts with its ALK receptor complex on caveolin lipid rafts to initiate GPI-anchor-receptor-independent endocytosis (133,149). Nodal may also interact with soluble convertases directly in the extracellular microenvironment. Once the pro-domain is cleaved, active Nodal can bind unidentified GPI-anchored receptors (besides Cripto) on the cell membrane to initiate endocytosis.
Within the cell, Nodal can be sequestered in intraluminal vesicles, to inhibit or delay signalling activity (134). Nodal trafficking has been studied using murine Nodal in an artificial expression system. Trafficking of endogenous human Nodal has not yet been defined. Furthermore, the requirement of Cripto in Nodal-mediated signalling in cancer has not yet been established. Regardless, this complex mode of intracellular trafficking intricately controls Nodal signalling via compartmentalization and degradation.

Nodal signalling range is determined by its stability, which is effected by cleavage of the Nodal N-terminal pro-domain by SPC1 and SPC4, and/or N-glycosylation (148). The presence of the N-terminal pro-domain normally functions to reduce uptake by endocytic compartments for protein trafficking (133,148). Studies by Constam and colleagues have shown that cleavage of the Nodal precursor in conditioned media triggers clearance within 24 hours via increased receptor-mediated uptake into endocytic vessels, and that intracellular mature-Nodal is degraded within 1 hour (148). Given that Nodal is an embryonic morphogen and therefore requires regulated signalling range, Constam has proposed that convertase processing is likely a mechanism for autocrine signalling (133,149). On the other hand, Nodal is stabilized by the presence of its pro-domain, and also by N-glycosylation, which is likely a mechanism for paracrine signalling (133,148). Constam has shown that N-glycosylation increases the stability of Nodal in conditioned media by reducing intracellular degradation (without affecting intracellular uptake) (148). Post-translational protein modifications are therefore important to maintain Nodal’s role as a
morphogen during embryonic patterning and development. Moreover, regulation of Nodal is very complex with numerous targets for aberrant activation.

The primary role of Nodal during embryonic development is to establish anterior-posterior axis patterning and left-right asymmetry (130). It is first expressed in the murine epiblast shortly after implantation, and is maintained and enhanced by autoregulation (154). Convertases expressed in the adjacent extraembryonic ectoderm process Nodal predominantly in the proximal epiblast (130,147). Activated Nodal signalling subsequently induces gene expression of Lefty1 (note: Lefty1 and Lefty2 are murine orthologs of human LeftyB and LeftyA, respectively) and CER1 in the distal visceral endoderm, which later becomes the anterior visceral endoderm (130). Nodal signalling is required for the displacement of the anterior visceral endoderm, determining the position of the anterior-posterior axis (130). Eventually, Nodal signalling becomes restricted by Lefty1 and CER1 to the proximal posterior region of the epiblast where the embryonic ectoderm and primitive endoderm are developing, and where the primitive streak will form (Figure 1.8) (131,133,139,155,156). Interestingly, signalling by a cleavage-resistant mutant of Nodal has been shown to induce mesoderm and EMT (133).

As development proceeds and cells undergo gastrulation, Nodal becomes restricted to the node at the anterior of the primitive streak, hence the name “Nodal” (130). The node initiates left-right axis formation (130,155,157,158). Nodal and growth differentiation factor 1 (GDF1) from the ventral node pattern the left side of the embryo through interactions with Cryptic in lateral plate
Figure 1.8 Nodal initiates anterior-posterior axis formation in mouse embryos. Nodal is activated in the proximal epiblast by convertase enzymes, such as Furin (SPC1) and PACE-4 (SPC4), from the extraembryonic ectoderm. Nodal is maintained through autoregulation, and eventually signals the distal visceral endoderm (DVE) to induce feedback inhibitors, such as Lefty1 and Cerberus-like (cerl). The DVE becomes the anterior visceral endoderm (AVE), whose displacement requires Nodal signalling in order to form the anterior-posterior axis. Lefty1 and cerl inhibit Nodal signalling in the anterior region of the embryo. Nodal therefore becomes restricted to the posterior region, where it plays a pivotal role in primitive streak formation.

Adapted from Schier, 2003.
mesoderm (139). On the right side of the embryo, Nodal inhibitors such as CER1 and Lefty1/2, and physical leftward flow from cilia restrict Nodal signalling (Figure 1.9) (139). During somitogenesis, Nodal becomes more specifically restricted to mesoderm cells on the left side of the embryo, and is downregulated with differentiation until it is no longer present at approximately 8 dpc (156).

Several studies with human embryonic stem cells have sought to elucidate the role of Nodal in human development. It is known that Nodal promotes pluripotency in human embryonic stem cells (159,160). Vallier and colleagues (2009) showed that Nodal signalling maintained pluripotency in human embryonic stem cells through SMAD2/3-induced activation of Nanog gene transcription (161). In turn, Nanog protein was shown to interact with SMAD2/3 to limit transcriptional activity of the Nodal signalling pathway, and inhibit endoderm differentiation (161). Several studies have also shown that inhibition of Activin/Nodal signalling in human embryonic stem cells by receptor inhibition with SB431542 induces neuroectoderm specification (161-163). Together, these studies exemplify the role of Nodal in maintaining pluripotency by inhibiting differentiation into neuroectoderm and mesendoderm lineages in human embryonic stem cells.

1.6.2 Nodal is expressed in placenta and dynamic adult tissues

As outlined, Nodal plays a primary role during embryonic development; however, there are a few (understudied) examples of Nodal-expressing extraembryonic and normal adult tissues, including the placenta, the involuting
Figure 1.9 Nodal initiates left-right axis formation in mouse embryos. Cilia-induced leftward flow within the embryo causes Nodal to localize to the left side of the node. Furthermore, Cerl and Lefty expression restrict Nodal to the left side of the node. On the left side of the embryo, Nodal signalling is facilitated by Gdf1 from the ventral node, and Cryptic, a Cripto-like co-receptor for Nodal in the lateral plate mesoderm. This results in a morphogenic gradient that inhibits Nodal in the right side of the embryo, and restrict its expression to the left side of the embryo and yield the left-right axis.

Adapted from Constam, 2009.
mammary gland, and the cycling endometrium. Interestingly, each of these systems is highly dynamic, and undergoes widespread remodelling events.

In the developing mammalian blastocyst, the trophectoderm cell layer surrounding the inner cell mass and blastocoel gives rise to the fetal portion of the placenta (164). Upon implantation, fetal trophoblast cells invade the maternal decidua and spiral arteries in order to gain access to the maternal blood supply. Given the role of Nodal in promoting invasive events during embryogenesis, recent studies have explored whether Nodal plays a role during placentation. In mice, insertional null \textit{Nodal} mutants exhibit disrupted embryonic development concomitant with abnormal placentation (165). Similarly, SPC1 and SPC4 double knockout mice show accelerated differentiation of trophoblast stem cells during placentation (166). \textit{In vitro} studies have demonstrated that Nodal-overexpression in HTR-8/SVneo trophoblast cells decreases cellular invasion and migration (167). Together these studies implicate a role for Nodal in mediating invasive trophoblast cell functionality both \textit{in vivo} and \textit{in vitro}.

As previously mentioned, during pregnancy and lactation, the terminal end buds of the mammary gland undergo proliferation and differentiation to form secretory milk-producing alveoli (14,15). Post-lactational involution reverts the mammary gland back to its pre-lactation state, and is characterised by widespread apoptosis of alveolar epithelial tissue, stromal remodelling, and adipocyte replenishment (9,168). Studies have found that Nodal and members of the Nodal signalling pathway are cyclically expressed during mammary gland remodelling. In particular, one study found that Nodal, Cripto, ALK-4, and SMAD4
were upregulated during lactation, and downregulated during involution in BalbC mice (137,169). These results suggest that Nodal signalling may play a dynamic role during mammary gland remodelling, whereby Nodal upregulation correlates with proliferative alveolar expansion, and Nodal downregulation correlates with apoptosis.

Nodal has also been studied in the human endometrium, which, like the mammary gland, undergoes many remodelling events during adulthood. Endometrium cycling consists of three phases: menstrual, proliferative (re-epithelialization), and secretory (170). Studies by Harrison and colleagues have studied Nodal signalling in human endometrium during the various phases of remodelling. His group has shown that Lefty, formerly known as Endometrial Bleeding-Associated Factor (EBAF), is highly expressed during late secretory and menstrual phases, and is downregulated during the proliferative phase (170). In contrast, Nodal is highly expressed throughout the proliferative phase and early secretory phase, and is abruptly downregulated by the mid-secretory phase (170). Again, although the endometrium harbours a completely different microenvironment compared to the mammary gland, it seems that Nodal expression correlates with proliferative epithelial expansion during endometrial cycling.
1.6.3 Nodal in Cancer

(See copyright release Appendix 1)

Nodal acts early in development, when the embryo consists of stem cell populations. Nodal is no longer expressed at later stages of development, when cells differentiate (130). Similar to its regulatory effects on embryonic stem cells, recent studies have suggested that Nodal is involved in maintaining the pluripotent phenotype of cancer cells, and that its actions result in increased cancer cell aggressiveness and tumourigenicity (127,131,171-173). When Nodal was inhibited with a small molecule inhibitory drug (SB431542) or morpholino oligonucleotides in melanoma cells, there was a marked reduction in tumour formation and metastasis, and a loss of cellular plasticity (127,172). This phenotypic transition occurred concomitant with an upregulation of Tyrosinase, and a downregulation of VE-Cadherin and Keratin 18, indicating a distinct genetic transition toward a more differentiated melanocyte-like cell type (127).

Similarly, one study reported a correlation between Nodal and invasive phenotypes in glioma (174). This group found that overexpression of Nodal in glioma cells that express low endogenous Nodal causes an increase in cellular invasion, MMP-2 secretion/activity, and proliferation in vitro, and tumour growth in vivo (174). Conversely, Nodal knockdown with shRNA in glioma cells that express high endogenous Nodal causes a decrease in cellular invasion, MMP-2 secretion/activity, and proliferation in vitro, and tumour growth in vivo (174). Importantly, this group found that Nodal expression is positively correlated with grade in human glioma tissue (174).
An article published in *Cell Stem Cell* by Heeschen and colleagues reported a correlation between Nodal and Activin signalling and pancreatic cancer. This study found that both Nodal and Activin were expressed at low levels in well-differentiated adherent pancreatic cancer cells, and at high levels in non-adherent pancreatic spheroids that express elevated pluripotency markers (called “cancer stem cells” (CSCs) in this study) (175). Treatment of CSCs with recombinant Nodal was found to increase spheroid formation, size, and invasion *in vitro* (175). Conversely, Activin/Nodal signalling inhibition in pancreatic cancer cells with an ALK4/7 inhibitor eliminated the CSC subpopulation and rendered cells receptive to gemcitabine chemotherapy *in vitro*, and in an orthotopic mouse model (175). Together, the results from this study demonstrate the robust effects of Nodal during disease progression and in promoting stem cell-like phenotypes in pancreatic cancer.

Nodal has been implicated in male-cancer types, such as testicular cancer and prostate cancer (176,177). Both Cripto and Nodal have been shown to be present in tumourigenic testicular cancer cells, such as NCCIT cells (177). One study by Adkins et al. (2003) demonstrated that inhibition of Cripto-Nodal signalling via an anti-EGF antibody (A27.F6.1) was able to inhibit tumour growth of NCCIT testicular cancer cells in nude mice (177). In prostate cancer, Nodal is correlated with invasive phenotypes (176). Compared to poorly aggressive LNCaP prostate cancer cells that express low levels of Nodal, aggressive DU145 prostate cancer cells express high levels of Nodal, and undergo anchorage-independent growth and invasion *in vitro* (176). Furthermore, transfection of
LNCaP cells with a Nodal expression vector increases clonogenicity in vitro (176).

Nodal signalling has also been implicated in female cancer types, such as endometrial cancer (170). As mentioned, the female endometrium undergoes constant remodelling and turnover during adult life, and Nodal has been positively correlated with proliferation during normal endometrial cycling (170). Interestingly, patient biopsies of endometrial carcinoma that ranged from Grade 1 to Grade 3 in severity showed a positive correlation between Nodal and Cripto expression, and cancer progression (170). Lefty, a potent inhibitor of Nodal signalling in embryonic stem cells, was absent in all endometrial cancer biopsies (170). These results are important for understanding normal mechanisms of proliferation in the endometrium, and aberrant mechanisms of endometrial carcinoma progression, in order to reveal new prognostic indicators of metastasis.

Several studies have linked Nodal with apoptosis in ovarian cancer (178). One study by Peng and colleagues from York University in Toronto found that Nodal overexpression in ovarian cancer cells was associated with decreased metabolic activity and proliferation (178). Although prima facie this finding seems to contradict the metastasis-promoting effects of Nodal in other forms of cancer, Peng’s experimental designs employ gain of function, over-expressing strategies (178,179). Overexpression of Nodal in cancer cells that already express Nodal tends to induce apoptosis, while overexpression of Nodal in cancer cells that do not normally express Nodal tends to promote metastasis. This trend is quite
interesting, as it highlights the concentration-dependent multi-functionality of Nodal signalling and timing in metastatic progression. This is not surprising, since other members of the TGF-β superfamily tend to have variable concentration-dependent effects during embryology and cancer progression, depending on temporal and spatial location (180-185).

Ongoing studies in our laboratory have demonstrated a role for Nodal in choriocarcinoma (186). Specifically, we have found that poorly-invasive BeWo cells have lower levels of Nodal protein compared to highly-invasive JAR and JEG-3 cells, and that Nodal protein expression is positively correlated with invasive capacity of these cell lines through Transwell chambers (186). In agreement with results from our laboratory, previous reports by Peng and colleagues have confirmed that Nodal and its receptors are present in BeWo, JAR and JEG-3 cells (167,179). However, in accordance with their results in ovarian cancer cell lines, Peng’s research group has reported that Nodal-overexpression in JAR and JEG-3 cell lines causes a decrease in proliferation and an induction of apoptosis (179), indicating that Nodal may elicits different effects in different contexts/conditions in this cancer type as well.

Recent studies have demonstrated a pro-metastatic role for Nodal and Cripto in breast cancer (131). Tissue microarray analyses of human breast tissue samples revealed a positive correlation between Nodal and breast cancer progression (187). Furthermore, Nodal was absent in normal breast tissue samples from these experiments. When MDA-MB-231 breast cancer cells were treated with Lefty from human embryonic stem cells, invasion and clonogenicity
was reduced concomitant with a downregulation of Nodal gene and protein expression (187).

Al-Hajj et al. identified breast cancer stem cells as CD44+/24-, which have since been used to demonstrate the plastic potential of aggressive breast tumour subpopulations (188). As mentioned, Meyer et al. was recently able to demonstrate that although CD44+/24- cells are accepted as the stem cell population within tumours, CD44+/24+ isolated populations are able to convert to CD44+/CD24- cells (and vise versa) both in vitro and in vivo, associated with a downregulation of E-Cadherin and an upregulation of stem cell associated genes, such as Slug (125). When isolated CD44+/24+ cell populations were transplanted into nude mice mammary fat pads, tumours were locally invasive, and did contain CD44+/24- populations upon examination (125). Interestingly, this suggests that differentiated cells outside the breast cancer stem cell subpopulation classically defined by Al-Hajj, exhibit a dynamic plastic phenotype. It was found that the central regulator of this dynamic phenotypic switching in CD44+/24+ and CD44+/24- cell populations was the Activin/Nodal pathway (125). When the ALK receptor was inhibited with SB431542 in either of these populations, phenotypic switching was significantly impaired, implying an important role for Nodal-associated signalling pathways in mediating plasticity in cancer (125). It is possible that Nodal is promoting these plastic phenotypes through mediating the transitions that occur during processes like EMT. Examining Nodal's normal role in maintaining embryonic pluripotency may lend insight towards its actions in cancer plasticity.
1.6.4 **Hypoxia promotes Nodal expression**

In light of the attention awarded to embryonic proteins in cancer, one important question is *how do cancer cells re-express embryonic proteins that are supposed to be silenced?* Oxygen is a microenvironmental factor that plays a pivotal role in regulating gene expression and cell fate during both embryological development and cancer progression. Interestingly, hypoxia constitutes both embryonic and tumour microenvironments, and mediates the activation of embryonic stem cell-associated programmes. HIF is the dominant mediator of the hypoxic response, and its fundamental nature is typified by its ubiquitous expression in nearly all cell types (189). Studies have shown that hypoxia promotes metastatic phenotypes including EMT, invasion and angiogenesis (83,190-192). These processes are mediated by an upregulation of factors such as TCF3 (which causes repression of E-Cadherin), VEGF (pro-angiogenic factor), and Twist (inducer of EMT) under hypoxic conditions (83,191,192).

Interestingly, our laboratory has recently shown that hypoxia causes upregulation of Nodal in breast and melanoma cell lines through HIF-1 (190). Specifically, HIF-1 was shown to bind to the NDE upstream of the Nodal gene locus, to promote transcription. Following Nodal upregulation, Nodal expression was shown to persist even after re-oxygenation, via an autoregulatory positive feedback loop. Although our work has uncovered a mechanism of Nodal expression in breast cancer cells, little is known about the role that Nodal plays during breast cancer progression after it has been re-expressed.
1.7 Hypothesis and Rationale

Given the role that Nodal plays in multiple types of cancer, and given that Nodal is re-expressed in breast cancer, the following study opts to resolve the question: What role does Nodal play during various aspects of the metastatic cascade? My hypothesis is that Nodal promotes metastatic phenotypes in breast cancer, including tumour growth, vascularization and invasion. Throughout my thesis, I have used multiple breast cancer cell lines: the highly-aggressive MDA-MB-231, MDA-MB-468, and Hs578t breast cancer cell lines, and the poorly-aggressive T47D and MCF-7 breast cancer cell lines. As will be evident in the following chapters, the highly-aggressive cell lines express high levels of Nodal and will be used for Nodal loss-of-function experiments, whereas the poorly-aggressive cell lines express relatively low levels of Nodal and will be used for Nodal gain-of-function experiments.

1.8 References


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CHAPTER 2

Nodal inhibition impairs breast cancer growth and progression
2.1 Abstract

Breast cancer progression is in part marked by the ability of tumour cells to exhibit uncontrolled growth. Tumours that display human embryonic stem cell (hESC)-associated gene expression signatures are more likely to progress than well-differentiated cancers and are thus associated with poor patient prognosis. Elevated proliferation and evasion of growth control are similarly associated with disease progression, and are classical hallmarks of cancer. In the current study we demonstrate that the hESC-associated factor, Nodal, promotes breast cancer growth. Specifically, we show that Nodal is elevated in aggressive MDA-MB-231, MDA-MB-468 and Hs578t human breast cancer cell lines, compared to poorly aggressive MCF-7 and T47D breast cancer cell lines. In a nude mouse model of tumourigenesis, Nodal knockdown via shRNA reduces tumour incidence and significantly blunts tumour growth at primary sites. In vitro, using Trypan Blue exclusion assays, Western blot analysis of phosphorylated histone H3 and cleaved caspase-9, and real time RT-PCR analysis of BAX and BCL2 gene expression, we demonstrate that Nodal promotes expansion of breast cancer cells, likely via a combinatorial mechanism involving increased proliferation and decreased apoptosis. In an experimental model of metastasis using beta-glucuronidase (GUSB)-deficient NOD/SCID/mucopolysaccharidosis type VII (MPSVII) mice, we show that although Nodal is not required for the formation of small (<100 cells) micrometastases at secondary sites, it supports an elevated proliferation:apoptosis ratio (Ki67:TUNEL) in micrometastatic lesions. Indeed, at longer time points (8 weeks), we determined that Nodal is necessary for the
subsequent development of macrometastatic lesions (100 cells). Our findings demonstrate that Nodal supports tumour growth at both primary and secondary sites by altering the ratio of proliferation to apoptosis in breast cancer cells. As Nodal expression is limited to embryonic systems and cancer, this study establishes Nodal as a potential tumour-specific target for the treatment of breast cancer.

2.2 Introduction

Tumour growth is dictated by elevated cellular proliferation and reduced apoptosis, to yield a net increase in cellular expansion. Accordingly, two classical and fundamental hallmarks of cancer include enhanced proliferation and evasion of apoptotic signals (1,2). Normally, epithelial cells require signals from their microenvironment to trigger entrance into a proliferative state. In contrast, cancer cells exhibit a reduced dependence on mitogenic factors from their microenvironment, and can enter a proliferative state in response to their own deregulated growth signals. In breast cancer, patients bearing tumours that express high levels of the proliferation marker nuclear antigen Ki67, concomitant with mutations in apoptotic programmes, exhibit accelerated disease progression and poor prognosis (3-6). Elucidating factors that regulate proliferative programmes and that, therefore, cause susceptibility to tumour cell expansion is of interest in order to develop effective targeted cancer therapies.

In addition to enhanced proliferation and evasion of apoptosis during cancer progression, aberrant expression of stem cell factors within breast
tumours has been shown to promote aggressive phenotypes, and is associated with growth-promoting profiles in tumour cells and their microenvironments. One example of a stem cell factor that is associated with cancer progression is Nodal, an embryonic morphogen and member of the Transforming Growth Factor-Beta (TGF-β) superfamily. Nodal expression is limited to pluripotent stem cells during embryonic development and to specialized dynamic adult tissue (such as the cycling endometrium), but is re-expressed to induce growth programmes in cancers such as melanoma, glioma, and prostate cancer (7-10). The role of Nodal during breast cancer progression has been minimally investigated; however, it has been reported that transient inhibition of Nodal in MDA-MB-231 breast cancer cells with morpholino oligonucleotides delays tumourigenesis in nude mice, concomitant with reduced proliferation (by Ki67 staining) and elevated apoptosis (by TUNEL staining) (11).

In accordance with its documented contribution to tumour growth, Nodal has recently been linked to proliferation in a variety of normal physiological systems. For example, Harrison and colleagues have studied Nodal signalling in human endometrium during the various phases of remodelling, and found that Nodal is highly expressed throughout the proliferative and early secretory phases, and is abruptly downregulated by the mid-secretory phase (12). In addition, Salomon and colleagues have found that Nodal and members of the Nodal signalling pathway are cyclically expressed during mammary gland remodelling. In particular, Nodal, Cripto, ALK4, and SMAD4 are upregulated during lactational expansion of alveolar epithelial tissue, and downregulated...
during involution (marked by widespread apoptosis) in BalbC mice (13,14). Together, these studies suggest that Nodal may play a role in promoting proliferative phenotypes in dynamic epithelial cell types.

Given the role of Nodal in promoting cancer progression, and that Nodal is correlated with proliferative adult tissues, we wanted to investigate the role of Nodal during breast cancer growth. First, we demonstrate that stable Nodal knockdown significantly blunts tumour growth in an orthotopic mouse model of tumourigenesis. *In vitro*, we found that Nodal promotes proliferation whilst preventing apoptosis in breast cancer cell lines. Overtime, we found that Nodal elevated the ratio of live:dead cells *in vitro*, indicating that this embryonic protein supports a net cellular expansion. Lastly, we evaluated the effect of Nodal on growth of secondary metastases. Using a unique experimental metastasis assay, we discovered that although Nodal does not affect the number of micrometastasis in the lung (i.e. seeding efficiency), it enhances proliferation:apoptosis ratios in micrometastases in favour of tumourigenic growth. Indeed, we found that Nodal is required for growth progression to macrometastases at secondary sites.

### 2.3 Methods

**2.3.1 Cell lines and treatments**

Two well-differentiated poorly-metastatic luminal-like breast cancer cell lines expressing low levels of Nodal (MCF-7 and T47D) and two highly-metastatic basal-like breast cancer cell lines expressing high levels of Nodal (MDA-MB-231...
and MDA-MB-468) were used for Nodal gain-of-function and Nodal loss-of-
function experiments, respectively. All cancer cell lines were obtained from the
American Type Culture Collection (ATCC) and were maintained as per
instructions. To increase Nodal signalling, we used a Nodal expression vector
(versus an empty pcDNA3.3 vector; pcDNA 3.3-TOPO cloning kit; Invitrogen).
To decrease Nodal signalling, we used Nodal-targeted shRNAs (versus
scrambled control shRNAs). Two Nodal-targeted shRNAs were used, a HuSH-
29mer (Id: GI311711; Origene) and a GIPZ lentiviral shRNAmir (Id:
V2LHS 155453; Open Biosystems) to rule-out off-target effects. Transfection
was performed with Arrest-In (Open Biosystems) or Lipofectamine (Invitrogen) as
per manufacturer instructions. For stable selection, Puromycin (200-450 ng/mL)
or Geneticin (G418; 800 ng/mL) was used.

2.3.2 RNA extraction and real-time PCR
RNA isolation was performed using the Perfect Pure RNA cultured cell kit (5
Prime), and DNase was used to degrade genomic DNA. Reverse transcription
was performed using 2 g of RNA and a High Capacity cDNA Reverse
Transcription kit (Applied Biosystems). Real-time PCR was performed with
TaqMan gene expression human primer/probe sets. For a list of primer/probes,
see Table 2.1. For analysis of Nodal receptor expression across cell types,
primer/probes included ALK4 (ACVR1B), ALK7 (ACVR1C), and Cripto-1
(TDGF1). Raw Ct values are presented, since housekeeping genes were often
different between cell lines and therefore delta Ct values misleadingly
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<tr>
<td>ALK7 (ACVR1C)</td>
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<td>BCL2</td>
<td></td>
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<tr>
<td>HPRT1</td>
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Table 2.1 Primer/Probe information for real time PCR
exaggerated differences. For analysis of BAX and BCL2 gene expression in response to treatments, Ct values were normalized to HPRT1, and compared using the ΔΔCt method.

2.3.3 Western blotting

Protein lysates were prepared using Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific) and Halt Protease Inhibitor Cocktail (Thermo Scientific) as per manufacturer’s instructions. Equal amounts of protein were reduced and separated by SDS-polyacrylamide gel electrophoresis, and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in 5% milk, incubated with primary antibody, washed with TBS-T 0.1% Tween, and incubated with horseradish peroxidase-conjugated secondary antibody. For a list of primary antibodies see Table 2.2. Enzyme activity was detected by enhanced chemiluminescence (Super Signal; Pierce). Images were obtained using film.

In accordance with previous studies (7,15-17), three banding locations were detected for Nodal: Pro-Nodal at 39 kDa, fully processed Nodal at 50 kDa, and mature Nodal at 15 kDa. The 50 kDa species is highly variable due to differences in post-translational modifications and protein lysate handling, and the 15 kDa band is typically in low abundance in both cell lysates and conditioned media due to low protein stability (16). For consistency, we used the 39 kDa band to assess Nodal expression in lysates, as we have previously shown that it is proportional to 15 kDa mature Nodal in both lysate and conditioned media (17).
<table>
<thead>
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<th>Primary Antibody</th>
<th>Clone</th>
<th>Company</th>
<th>Dilution</th>
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<tbody>
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<td>WS65</td>
<td>Santa Cruz</td>
<td>1:500</td>
</tr>
<tr>
<td>Monoclonal rabbit anti-phospho-SMAD2</td>
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<td>Millipore</td>
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</tr>
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<td>Santa Cruz</td>
<td>1:5000</td>
</tr>
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<td></td>
<td>Cell Signalling</td>
<td>1:1000</td>
</tr>
<tr>
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Table 2.2 List of primary antibodies used for Western blot analyses.
2.3.4 Tumour assay in nude mice

Two nude mouse models (Crl:NU-\textit{Foxn1}^{nu}; Charles River) were used to evaluate the effects of Nodal knockdown (in highly aggressive MDA-MB-468 or MDA-MB-231 cells) on tumourigenesis \textit{in vivo}. All experiments involving animals were approved by the Animal Use Subcommittee at the University of Western Ontario, Protocol No. 2008-101 (Appendix 2).

**Flank tumour assay:** MDA-MB-468 cells were transfected with a Control HuSH shRNA, or a Nodal-targeted HuSH shRNA and stably selected with Puromycin. 2,500,000 cells in 100 L of RPMI + Matrigel (1:1) were injected into the right flank of 6-8 week old female mice. Twice per week, the longest dimension of the tumour that could be seen through the mouse skin was measured using a digital caliper. We choose to use only one dimension as an \textit{indication} of growth rate, since the volume of an irregularly shaped tumour cannot be accurately calculated until it is excised and the depth can be quantified. Of note, following tumour excision, the total volume of the tumour was calculated by measuring all three growth dimensions (length x width x depth).

**Orthotopic tumour assay:** MDA-MB-231 cells were transfected with a Control GIPZ shRNA or a Nodal-targeted GIPZ shRNA, and stably selected with Puromycin. 500,000 cells in 50 L of RPMI were injected into the mammary fat pad of 6-8 week old female mice. Twice per week, the longest dimension of the tumour that could be seen through the mouse skin was measured using a digital
caliper. Following tumour excision, the total volume of the tumour was calculated (length x width x depth).

2.3.5 In vitro growth curves and cell death analyses

Cells were seeded into 6-well plates (100,000 cells/well) and counted over 3 days. Media containing dead and live cells was collected. Attached cells were harvested using Trypsin, combined with media, spun down, and resuspended with Trypan Blue to demarcate non-viable cells. A Countess automated cell counter (Invitrogen) was used to calculate total cell number, live cells, and dead cells. Growth curves and Live:Dead ratios were calculated from this data.

2.3.6 Experimental metastasis assay in NOD/SCID/MPSVII mice

500,000 cells in 700 μL Ca\(^{2+}\)-free HBSS were injected into the tail vein of NOD/SCID/MPSVII mice. Mice were sacrificed at 4 weeks (to assess micrometastases) or 8 weeks (to assess macrometastases). Lung, brain, and liver from transplanted NOD/SCID/MPSVII mice were either frozen in OCT embedding medium (Sakura Finetek, Torrance, CA) for histochemical analysis of GUSB activity, or were fixed in 4% formaldehyde and paraffin-embedded for analysis of TUNEL and Ki67:

GUSB staining and assessment of metastases from frozen tissue:

Frozen serial sections of 10 μm thickness were fixed with 10% buffered formalin (Sigma Aldrich, St. Louis, MO), and blocked with mouse-on-mouse reagent (Vector Laboratories, Burlingame, CA). Sections were analyzed for human cells
by colourimetric detection of ubiquitous GUSB activity in human cells as previously described using napthol AS-BI β-D-glucuronide substrate (Sigma-Aldrich) (18), and counterstained with haematoxylin. Within one section, metastases that were <100 cells were considered micro’, while metastases that were 100 cells were considered macro’. Micrometastases versus macrometastases were counted manually under a microscope. It should be noted that, usually, micrometastases were much less than 100 cells, and macrometastases were much more than 100 cells, and therefore, differentiating between these two types of lesions was clear. For each mouse organ, 3-6 sections were acquired from evenly spaced areas through the tissue, and the average number of metastases per mouse organ (i.e. per 3 sections) was calculated.

**Ki67 and TUNEL staining and assessment of Proliferation:Apoptosis from paraffin-embedded tissue:** Serial sections of 4 μm thickness were obtained, and immunohistochemical staining was conducted using a human-specific Ki67 antibody (Monoclonal rabbit anti-hKi67, clone SP6; Ready to use; Thermo Scientific) as per manufacturer’s instructions. The DeadEnd colorimetric TUNEL system (Promega) was used to measure apoptosis as per instructions. The proliferation:apoptosis ratio was determined by counting Ki67-positive and TUNEL-positive nuclei in matched serial sections. At least 3 pairs of serial sections, evenly spaced through the tissue, were averaged per mouse to yield one proliferation-to-apoptosis score for that animal.
2.3.7 Statistical analyses

Statistics were performed using SigmaStat (Dundas Software), and validated through the biostatistical support unit at the University of Western Ontario. All parametric data was analysed using a one-way ANOVA and a Tukey Kramer Comparisons Post-Hoc test, and expressed as mean S.E.M. for replicate values. All non-parametric data was analyzed using an ANOVA on Ranks followed by the Mann-Whitney rank-sum test, and expressed as median interquartile range. A student's t-test was used to compare two items. All statistical tests were two-sided, and data were considered statistically significant at $p<0.05$.

2.4 Results and Discussion

2.4.1 Nodal is elevated in aggressive (c.f. poorly aggressive) breast cancer cell lines

Through Western blot analyses, we determined that Nodal protein is elevated in poorly-differentiated, basal-like, metastatic Hs578t, MDA-MB-231 and MDA-MB-468 breast cancer cell lines compared to well-differentiated luminal-like MCF-7 and T47D cell lines (Figure 2.1A) (19). This is consistent with previous reports that show high Nodal expression in aggressive melanoma, prostate, and breast cancer cell lines compared to poorly aggressive lines, supporting the hypothesis that Nodal expression is elevated during cancer progression (9,15).

Nodal signals through interactions with Cripto-1 (TDGF1) and the Activin-Like Kinase type I (ALK4/7) and type II (ActRIIB) receptor complex. Activation of
A

<table>
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<th>kDa</th>
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</tr>
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</table>

B

![Graph showing Raw Ct value for different cell lines and gene expressions.](image-url)
Figure 2.1 Nodal is associated with aggressive breast cancer cells.
(A) Western blot analysis of Nodal in poorly-differentiated (MDA-MB-231, MDA-MB-468, Hs578t) and well-differentiated (MCF-7, T47D) breast cancer cells. Nodal expression is elevated in highly aggressive MDA-MB-231, MDA-MB-468 and Hs578t breast cancer cells compared to poorly aggressive MCF-7 and T47D breast cancer cells. ~39 kDa Pro-Nodal band is depicted and Actin is used as a loading control. (B) Raw Ct values from real time RT-PCR analysis of equal amounts of cDNA demonstrating that all members of the Nodal receptor complex, including ALK-4, ALK-7 and Cripto (TDGF1), are expressed in MDA-MB-231, MDA-MB-468, Hs578t, MCF-7 and T47D breast cancer cell lines (n=4). Lower Ct values are indicative of exponentially higher levels of expression.
this receptor complex leads to SMAD2/3 phosphorylation, and subsequent Nodal-mediated gene expression (20). It has been reported that Nodal receptor components are expressed at varying levels in prostate cancer cell lines (10). Similarly, we wanted to determine whether the cell lines used in this study expressed Nodal receptor components, in order to ensure our cell lines had the potential to respond to treatment conditions. Using real time RT-PCR, we determined that all members of the Nodal receptor complex, including ALK4, ALK7 and TDGF1, are expressed in Hs578t, MDA-MB-231, MDA-MB-468, T47D and MCF-7 breast cancer cell lines at varying levels (Figure 2.1B). This suggests that these cell lines are able to respond to and carry out Nodal-induced signal transduction, providing a context upon which to study both Nodal gain-of-function and Nodal loss-of-function effects.

2.4.2 Nodal promotes breast cancer tumourigenesis

Given that Nodal is associated with aggressive cancers and breast cancer cell lines, we first sought to determine whether stable Nodal knockdown regulates breast cancer tumourigenesis in vivo. Previous studies demonstrated that transient inhibition of Nodal with morpholinos or exposure to its antagonist, Lefty, diminished tumour initiation in breast cancer and melanoma models (7,15). In order to better understand the role of Nodal in tumour growth over an extended period of time, we stably knocked down Nodal expression in aggressive MDA-MB-468 and MDA-MB-231 breast cancer cells using puromycin-selectable shRNAs. In our first model, we injected 2.5 million MDA-MB-468 cells transfected
with a Control shRNA (468+shControl) or a Nodal-targeted shRNA (468+shNodal) into the flanks of nude mice, and measured tumour growth over 6 weeks. This approach revealed that Nodal knockdown significantly impaired MDA-MB-468 tumour growth, and resulted in a 2-fold reduction in tumour volume following excision (p<0.05) (Figure 2.2A-C).

As a corollary to this experiment, in our second model, we injected 500,000 MDA-MB-231 cells transfected with a Control shRNA (231+shControl) or Nodal-targeted shRNA (231+shNodal) through the nipple into the mammary fat pad of nude mice, and measured tumour growth over 9 weeks. Compared to the flank model, this model was more stringent since we injected fewer cells (0.5 versus 2.5 million), Matrigel was not used to help tumours initiate, and the breast cancer cells were injected into the mammary environment to recapitulate a relevant physiological context. We found that Nodal knockdown significantly impaired tumour growth compared to controls (p<0.05, n=10) (Figure 2.2D,E). Furthermore, there was a 5-fold reduction in tumour volume following excision (p<0.05) (Figure 2.2F). Importantly, we observed a phenomenon that was not apparent in our flank model. We found that unlike 231+shControl tumours which continued to grow over time, the 231+shNodal tumours experienced a plateau in growth at a diameter of approximately 1.5 mm. This suggested to us that Nodal inhibition may alter cell viability, proliferation or death to counteract tumour growth. It should be noted that Nodal knockdown in the MDA-MB-231 cell line was more robust than knockdown in the MDA-MB-468 cell line (by Western blot
**A**

KDa

<table>
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<td>Actin</td>
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MDA-MB-468

**C**

Tumour Volume (mm³)

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**D**

KDa

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MDA-MB-231

**F**

Tumour Volume (mm³)

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<th>shRNA Nodal</th>
</tr>
</thead>
</table>

**B**

Tumour Diameter (mm)

Week

| 468+shControl | 468+shNodal |

**E**

Tumour Diameter (mm)

Week

| 231+shControl | 231+shNodal |
Figure 2.2 Nodal promotes tumorigenesis in vivo. (A) Western blot confirming Nodal knock-down by shRNA in MDA-MB-468 cells. The ~39 kDa Pro-Nodal band is presented and Actin is used as a loading control. (B) 2.5 million MDA-MB-468 cells transfected with Control shRNA (468+shControl) or a Nodal-targeted shRNA (468+shNodal) were injected with Matrigel into the flanks of nude mice, and tumor diameter was measured over the course of 6 weeks. 468+shControl cells formed significantly larger tumors compared to 468+shNodal cells (n=8, p<0.05). Values represent mean tumor diameter (mm) ± S.E.M. (C) Tumor volume of MDA-MB-468-derived tumors excised after 6 weeks. Bars represent mean tumor volume (mm$^3$) ± S.E.M. (D) Western blot confirming Nodal knock-down by shRNA in MDA-MB-231 cells. The ~39 kDa Pro-Nodal band is presented and Actin is used as a loading control. (E) 0.5 million MDA-MB-231 cells transfected with Control shRNA (231+shControl) or Nodal-targeted shRNA (231+shNodal) were orthotopically injected into the mammary fat pads of nude mice, and tumor diameter was measured over the course of 9 weeks. 231+shControl cells formed significantly larger tumors compared to 231+shNodal cells (n=10, p<0.05). Values represent mean tumor diameter (mm) ± S.E.M. (F) Tumor volume of MDA-MB-231-derived tumors excised after 9 weeks. Bars represent mean tumor volume (mm$^3$) ± S.E.M.
validation in Figure 2.2A compared to Figure 2.2D). This may also explain why the plateau effect was not apparent in the flank model.

2.4.3 The effects of Nodal on proliferation in vitro

Given our finding that Nodal inhibition causes a reduction in tumour growth in vivo, we wanted to examine the effects of Nodal on cell proliferation in vitro. We used Trypan Blue exclusion assays to generate growth curves for breast cancer cells in response to alterations in Nodal expression. As a Nodal gain-of-function model, we used T47D cells stably transfected with a Nodal expression construct (T47D+Nodal) versus an empty vector control (T47D+EV) (Figure 2.3A). We found that T47D+Nodal cells displayed a significant increase in proliferation after 3 days compared to T47D+EV cells (n=3; p=0.046) (Figure 2.3B). As a Nodal loss-of-function model, we compared growth curves for 468+shNodal cells versus 468+shControl cells, or 231+shNodal cells versus 231+shControl cells. We found that Nodal inhibition by shRNA significantly reduced proliferation in MDA-MB-468 cells (n=3; p=0.046) and in MDA-MB-231 cells (n=3, p=0.047) compared to controls after 3 days (Figure 2.3C,D). In accordance with these results, Western blot analyses confirmed that histone H3 phosphorylation at 4 different sites, including Ser10, Ser28, Thr3, and Thr11, was lower in 231+shNodal cells compared to 231+shControl cells, indicative of reduced mitosis (Figure 2.3E). In general, although the growth curves showed changes in response to altered Nodal expression, the changes were small and likely do not solely account for the robust effects seen on tumour growth in vivo.
Cellular Proliferation (Fold change rel. to Day 1)

Day

T47D+EV  T47D+Nodal

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0

Cellular Proliferation (Fold change rel. to Day 1)

Day

468+shControl  468+shNodal

0.0 1.0 2.0 3.0 4.0 5.0 6.0

Cellular Proliferation (Fold change rel. to Day 1)

Day

231+shControl  231+shNodal

0.0 1.0 2.0 3.0 4.0

Cellular Proliferation (Fold change rel. to Day 1)

Day

kDa

37- 42-

Nodal  Actin

Nodal Ac/g415n 37-

42-

P-Histone H3 (Ser10)  P-Histone H3 (Ser28)  P-Histone H3 (Thr3)

Histone H3  Actin

P-Histone H3 (Thr11)

17-

17-

17-

17-

17-

17-

42-
Figure 2.3 Nodal promotes proliferation of breast cancer cells in vitro. (A) Western blot validating elevated Nodal expression in T47D cells transfected with a Nodal expression vector (T47D+Nodal) versus an empty vector control (T47D+EV). (B-D) Trypan Blue exclusion was used to count live cells daily to generate growth curves over 3 days, in response to altered Nodal expression. (B) T47D+Nodal cells exhibited a significant increase in proliferation compared to T47D+EV cells over 3 days (n=3, p=0.046). (C) MDA-MB-468 cells transfected with a Nodal-targeted shRNA (468+shNodal) exhibited a significant decrease in proliferation over 3 days compared to cells transfected with a scrambled Control shRNA (468+shControl) (n=3; p=0.046). (D) MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) exhibited a significant decrease in proliferation over 3 days compared to cells transfected with a scrambled Control shRNA (231+shControl) (n=3; p=0.047). (E) Western blot demonstrating decreased phosphorylated histone H3 at 4 different sites, including Thr11, Ser10, Ser28 and Thr3 in 231+shNodal cells compared to 231+shControl cells. Total histone H3 and β-Actin are used as controls. All data are presented as mean S.E.M. for replicate values. Asterisks indicate a significant difference compared to controls.
2.4.4 The effects of Nodal on apoptosis in vitro

Given that Nodal had a small effect on proliferation in vitro, we hypothesized that perhaps Nodal could also regulate apoptotic phenotypes, which might help to explain the large differences observed during in vivo tumour growth. In order to explore the effects of Nodal on apoptosis, we performed Western blot analyses for activated (cleaved) caspase-9 in T47D+EV cells versus T47D+Nodal cells. We found that cleaved caspase-9 was present at lower levels in T47D+Nodal cells compared to controls, indicative of reduced apoptosis in the presence of Nodal (Figure 2.4A). Given that caspase-9 is frequently associated with mitochondria-mediated apoptosis (refer to Section 1.4.1.3), we decided to quantify BAX and BCL2 mRNA expression in T47D+EV cells versus T47D+Nodal cells. Real time RT-PCR analysis indicated that there was a significant decrease in BAX expression (n=5, p=0.016) and a significant increase in BCL2 expression (n=5, p=0.016) in T47D+Nodal cells compared to controls (Figure 2.4B,C).

As a corollary, caspase-9 cleavage, and BAX and BCL2 gene expression were measured in 231+shNodal versus 231+shControl cells. We found that cleaved caspase-9 was present at higher levels in 231+shNodal cells compared to controls, indicative of elevated apoptosis in the absence of Nodal (Figure 2.4D). Consistently, real time RT-PCR indicated that there was a significant increase in BAX expression (n=4, p=0.029) and a significant decrease in BCL2
A. Cleaved Caspase-9 and Caspase-9 expression levels.

B. Graph showing BAX mRNA expression (Fold change rel. to T47D+EV).

C. Graph showing BCL2 mRNA expression (Fold change rel. to T47D+EV).

D. Cleaved Caspase-9 and Caspase-9 expression levels.

E. Graph showing BAX mRNA expression (Fold change rel. to 231+shControl).

F. Graph showing BCL2 mRNA expression (Fold change rel. to 231+shControl).
Figure 2.4 Nodal reduces apoptosis of breast cancer cells in vitro.

(A) Western blot demonstrating that cleavage of caspase-9 is reduced in T47D cells transfected with a Nodal overexpression construct (T47D+Nodal) compared to cells transfected with an empty vector control (T47D+EV). Uncleaved caspase-9 and β-Actin are used as controls. (B) Real time RT-PCR analysis demonstrating that BAX mRNA expression is significantly lower in T47D+Nodal cells compared to T47D+EV cells (n=5, p=0.016). (C) Real time RT-PCR analysis demonstrating that BCL2 mRNA expression is significantly higher in T47D+Nodal cells compared to T47D+EV cells (n=5, p=0.016). (D) Western blot demonstrating that cleavage of caspase-9 is elevated in MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) compared to cells transfected with a scrambled Control shRNA (231+shControl). Uncleaved caspase-9 and β-Actin are used as controls. (E) Real time RT-PCR analysis demonstrating that BAX mRNA expression is significantly higher in 231+shNodal cells compared to 231+shControl cells (n=4, p=0.029). (F) Real time RT-PCR analysis demonstrating that BCL2 mRNA expression is significantly lower in 231+shNodal cells compared to 231+shControl cells (n=4, p=0.029). All data are presented as mean ± S.E.M. for replicate values. Asterisks indicate a significant difference compared to controls.
expression (n=4, p=0.029) in 231+shNodal cells compared to controls (Figure 2.4E,F).

2.4.5 The effects of Nodal on Live:Dead cell ratios in vitro

As previously mentioned, tumour growth is dictated by elevated cellular proliferation and reduced apoptosis, to yield a net increase in cellular expansion (1,2). Therefore, the ratio of live:dead cells at any given time is an important indicator of growth potential. Given our findings that Nodal affects both proliferation and apoptosis in 3 day-old cultures, we wanted to verify that Nodal did indeed have an effect on the overall ratio of live:dead cells at this time. As a Nodal gain-of-function model, we found that T47D+Nodal cells exhibited a significant increase in the ratio of live:dead cells after 3 days compared to T47D+EV cells (n=3, p=0.038) (Figure 2.5A). As a Nodal loss-of-function model, we compared live:dead cell ratios for 468+shNodal cells versus 468+shControl cells, or 231+shNodal cells versus 231+shControl cells. We found that after 3 days, 468+shNodal cells exhibited a significant decrease in the ratio of live:dead cells (n=3; p=0.033) compared to 468+shControls (Figure 2.5B). Consistently, 231+shNodal cells exhibited a significant decrease in the ratio of live:dead cells (n=3, p=0.024) compared to 231+shControls (Figure 2.5C).

Taken together, our results demonstrate that Nodal promotes elevated net growth in culture. This in part explains the observation that Nodal inhibition blunts tumour growth in vivo. Interestingly, it has been reported that Notch4, which
A

![Graph A](A.png)

Live:Dead Ratio
(Fold change rel. to T47D+EV)

T47D+EV  T47D+Nodal

* *

B

![Graph B](B.png)

Live:Dead Ratio
(Fold change rel. to 468+shControl)

468+shControl  468+shNodal

*

C

![Graph C](C.png)

Live:Dead Ratio
(Fold change rel. to 231+shControl)

231+shControl  231+shNodal

*
Figure 2.5 Nodal promotes an elevated Live:Dead cell ratio in breast cancer cells *in vitro*. Trypan blue exclusion was used to calculate the Live:Dead cell ratio of breast cancer cell lines after 3 days in culture, in response to altered Nodal expression. (A) T47D cells transfected with a Nodal overexpression construct (T47D+Nodal) exhibited an elevated Live:Dead cell ratio after 3 days compared to T47D cells transfected with an empty vector control (T47D+EV) (n=3, p=0.038). (B) MDA-MB-468 cells transfected with a Nodal-targeted shRNA (468+shNodal) exhibited a decreased Live:Dead cell ratio after 3 days compared to cells transfected with a scrambled Control shRNA (468+shControl) (n=3; p=0.033). (C) MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) exhibited a decreased Live:Dead cell ratio after 3 days compared to cells transfected with a scrambled Control shRNA (231+shControl) (n=3, p=0.024). All data are presented as mean ± S.E.M. for replicate values. Asterisks indicate a significant difference compared to controls.
regulates Nodal expression in melanoma models, promotes proliferation and inhibits apoptosis in C8161, MV3, and SK-MEL-28 melanoma cell lines (21). Furthermore, Nodal over-expression in GBM glioma cells causes an increase in proliferation concomitant with elevated tumourigenesis in mice (8). In contrast to the results shown here, studies by Peng and colleagues have reported that overexpression of Nodal promotes apoptosis and inhibits proliferation in MDA-MB-231 breast cancer cell lines (22,23). However, one key difference in Peng’s experimental design is that Nodal was over-expressed in MDA-MB-231 cells (which express high endogenous Nodal), whereas here, Nodal was inhibited in MDA-MB-231 cells. Furthermore, the dose of recombinant Nodal that was used in Peng’s study was 500 ng/mL, which is more than 5-fold higher than the dose of Nodal necessary to sustain its role in maintaining pluripotency in hESC cultures (23-25). This brings light to the possibility that Nodal exhibits a concentration-dependent biphasic effect on breast cancer progression, similar to the function of TGF-β (26,27).

**2.4.6 Nodal promotes growth from micro to macrometastases**

Cancer becomes a fatal disease once it has metastasized and grown into a sufficient secondary tumour mass. However, the metastatic cascade is a highly inefficient process overall, and it has been reported that one of the most inefficient steps is growth at the secondary site (28,29). Given that Nodal inhibition causes a plateau in primary tumour growth *in vivo*, and that it alters
proliferation and cell death in vitro, we opted to test the effect of Nodal inhibition on secondary tumour growth.

Accordingly, we developed a model that takes advantage of a simple experimental metastasis assay using NOD/SCID/mucopolysaccharidosis type VII (NOD/SCID/MPSVII) mice, which are deficient in the lysosomal enzyme beta-glucuronidase (GUSB) (18). The GUSB model allowed us to attain single-cell resolution of transplanted human tumour cells by virtue of their constitutive GUSB activity within the GUSB-deficient mouse, thereby enabling the identification of lesions down to single-cell level that would be undetectable using conventional histology. We sacrificed mice at two different time points, at 4 weeks and 8 weeks, following tail vein injection of 231+shControl or 231+shNodal cells. At both 4 and 8 weeks, brain and liver tissue were also evaluated for evidence of metastasis (Figure 2.6). Using this high-resolution experimental metastasis model, we found only micrometastases of <100 cells at 4 weeks post-injection, and discovered that Nodal knockdown did not cause a significant change in the number of micrometastases that formed in the lung (Figure 2.7A,B). This suggested that Nodal does not affect seeding at secondary sites.

In addition to seeding, another important step in the metastatic cascade is growth at the secondary site. Indeed, metastatic tumour cells can reside and survive at secondary sites in the body while circumventing a need for growth or progression; an aspect of carcinogenesis called tumour dormancy. Tumour mass dormancy, in particular, refers to metastases that remain asymptomatic due to an inability to expand in size, and is often attributed to a counterbalance of
Tail vein injection (500,000 cells)

**Micrometastasis**
- 4 weeks: Sacrifice mouse
  Collect Brain, Lung, Liver
  - Flash Freeze
  - GUSB
  - Paraffin-Embed
  - H&E, TUNEL, Ki67

**Macrometastasis**
- 8 weeks: Sacrifice mouse
  Collect Brain, Lung, Liver
  - Flash Freeze
  - GUSB
  - Paraffin-Embed
  - H&E
Figure 2.6 Experimental design for examination of the transition from micro- to macro-metastases. Schematic depicting experimental process for GUSB metastasis model. Briefly, MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) or a Control shRNA (231+shControl) were injected through the tail vein of NOD/SCID/MPSVII mice, which exhibit GUSB deficiency. After 4 weeks, micrometastasis formation from human cells was assessed in the lung, liver and brain of animals, marked by positive GUSB enzymatic staining. At 8 weeks, macrometastasis formation from human cells was assessed in the lung, liver and brain of animals, by GUSB enzymatic staining and H&E. Ki67 and TUNEL were used to assess proliferation:apoptosis ratios.
A

231+shControl

231+shNodal

B

Number of Micromets @ 4 weeks
(Average of 3 lung sections/mouse)

C

Proliferation: Apoptosis Index

D

TUNEL

ki67

231+shControl

231+shNodal
Figure 2.7 Nodal inhibition alters proliferation-to-apoptosis ratios in micrometastases. (A) GUSB staining of pulmonary micrometastases from MDA-MB-231 cells transfected with a Control shRNA (231+shControl) or a shRNA to Nodal (231+shNodal) in NOD/SCID/MPSVII mice 4 weeks post-intravenous injection (red and outlined with white dotted line). (B) Scatter plot representing the average number of micrometastases (<100 cells) per section of lung from NOD/SCID/MPSVII mice 4 weeks after injection with 231+shControl or 231+shNodal cells. The number of 231+shNodal micrometastases that formed after 4 weeks in NOD/SCID/MPSVII mice was not significantly reduced compared to the number of 231+shControl micrometastases (n≥5, p 0.05). Each point represents the average mean number of micrometastases per section per mouse. Black bars represent the median number of micrometastases per section per mouse. (C) Immunohistochemical analysis of Ki67 expression (brown) and TUNEL (brown) staining in pulmonary micrometastases from 231+shControl cells or 231+shNodal cells in NOD/SCID/MPSVII mice 4 weeks post-intravenous injection. Proliferation is indicated by Ki67 staining and apoptotic nuclei were detected with TUNEL. (D) Proliferation:apoptosis ratios in 4 week micrometastases were determined with immunohistochemical localization of Ki67 and TUNEL. At 4 weeks, lesions from 231+shControl cells had a positive proliferation ratio (1.57) whereas lesions from 231+shNodal cells had a negative proliferation:apoptosis ratio (0.74) (n≥3, p<0.05). Values represent mean average proliferation:apoptosis ratio in tumor lesions per mouse S.E.M.
proliferation and apoptosis (reviewed in (30)). Dormant tumours are often not dangerous; however, their potential to overcome their dormant state poses a threat to patient health. Accordingly, we measured proliferation:apoptosis ratios in the 4-week micro-lesions via immunohistochemical staining for Ki67:TUNEL. We found that 231+shControl lesions had a proliferation:apoptosis ratio greater than 1, indicating a potential for tumour growth, whereas 231+shNodal lesions had a proliferation:apoptosis ratio less than 1, indicating a state of tumour mass dormancy or regression (Figure 2.7C,D).

Given these results, we expected that tumours that exhibited a potential for growth at 4 weeks would progress to macrometastases by 8 weeks, and far exceed the 100-cell limit observed in the 4-week lesions. Of note, by 8 weeks the 231+shControl lesions formed overt pulmonary macrometastases in all of the mice injected with these cells (Figure 2.8A,B). In contrast, the 231+shNodal cells did not form macrometastases. Rather, at 8 weeks there was a significant accumulation of micrometastases in the lung compared to 231+shNodal cells at the 4 week time point (n≥8, p<0.05) (Figure 2.8C). We also detected metastases in the brain of 1/5 231+shControl injected mice at 4 weeks and in the liver of 1/4 231+shControl injected mice at 8 weeks (Figure 2.9). However, metastases to the brain or the liver were not detected in any of the 16 231+shNodal-injected mice.

Taken together, the results from our experimental metastasis assay illustrate the importance of Nodal in regulating the transition between micrometastatic and macrometastatic growth, in part through its ability to alter
A 231+shControl  231+shNodal

B 231+shControl  231+shNodal

C

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*
Figure 2.8 Nodal promotes progression to macrometastases. (A) H&E staining demonstrates macrometastasis formation after 8 weeks post-intravenous injection in NOD/SCID/MPSVII mice for 231+shControl cells, but not 231+shNodal cells. 100% of lungs seeded with 231+shControl cells (4/4) contained macroscopic lesions at 8 weeks, whereas 0/8 lungs seeded with 231+shNodal cells contained macroscopic lesions. (B) GUSB staining to confirm human origin of lesions showing 231+shControl macrometastasis formation after 8 weeks. Although macrometastases were not detected in mice injected with 231+shNodal cells, micrometastases were detected with GUSB staining. (C) The number of 231+shNodal micrometastases that formed after 8 weeks in NOD/SCID/MPSVII mice was significantly higher than those that formed after 4 weeks (n=8, p<0.05). Bars represent the average mean number of micrometastases per section per mouse ± S.E.M.
A  231+shControl 4 weeks (Brain)

B  231+shControl 8 weeks (Liver)
Figure 2.9 Nodal supports tumour metastasis. (A) GUSB staining of a brain metastasis from MDA-MB-231 cells transfected with a Control shRNA (231+shControl) in NOD/SCID/MPSVII mice 4 weeks post-intravenous injection (red). (B) GUSB staining demonstrates a liver macrometastasis from 231+shControl cells after 8 weeks. No tumours were found in either brain or liver from 231+shNodal cells at either 4 or 8 weeks.
proliferation: apoptosis ratios necessary for normal tissue homeostasis. Similar phenomena have been reported in C57BL6/J mouse models of Lewis lung carcinoma mice, whereby poor tumour vascularization caused tumour dormancy marked by equal rates of mitosis and apoptosis (31). Interestingly, previous findings from our laboratory have implicated Nodal in regulating tumour angiogenesis both in vitro and in vivo (32). Thus, in addition to directly regulating cell proliferation and/or apoptosis, Nodal may indirectly promote tumour growth by facilitating vascular recruitment, a possibility worth investigating in future studies (see Chapter 3). Finally, although Nodal did not affect seeding of the cells at the secondary site, micrometastases accumulated over time with Nodal-deficient cells. Conventional tumour growth assays using histology and/or whole animal imaging do not permit the single cell resolution that we obtained with the GUSB model; hence, studies done using conventional methodologies may inadvertently overlook the seeding phenomenon uncovered here.

Collectively, this study indicates that the stem cell-associated protein Nodal promotes breast cancer tumour growth at both primary and secondary tumour sites, by altering the balance between proliferation and apoptosis. Our results provide mechanistic insight into studies that demonstrate that cancer cells manifesting stem-cell like properties exhibit accelerated cancer growth and progression in vivo, compared to their well-differentiated counterparts (33,34). Since Nodal expression is limited to embryonic contexts, our discovery suggests a novel role for Nodal as a tumour-specific target against breast cancer.
progression, and for maintenance of tumour dormancy following metastatic spread.

2.5 References


CHAPTER 3

The embryonic protein Nodal promotes breast cancer vascularization
3.1 Abstract

Tumour vascularization is requisite for breast cancer progression, and high microvascular density in tumours is a poor prognostic indicator. Patients bearing breast cancers expressing human embryonic stem cell (hESC)-associated genes similarly exhibit high mortality rates, and the expression of embryonic proteins is associated with tumour progression. Here, we demonstrate that Nodal, a hESC-associated protein, promotes breast cancer vascularization. We show that high levels of Nodal are positively correlated with high vascular densities in human breast lesions (p=0.0078). In vitro, we demonstrate that Nodal facilitates breast cancer-induced endothelial cell migration and tube formation, largely by up-regulating the expression and secretion of pro-angiogenic factors by breast cancer cells. Using a directed in vivo angiogenesis assay, and a chick chorioallantoic membrane assay, we show that Nodal promotes vascular recruitment in vivo. In a clinically relevant in vivo model whereby Nodal expression was inhibited following tumour formation, we found a significant reduction in tumour vascularization concomitant with elevated hypoxia and tumour necrosis. These findings establish Nodal as a potential anti-angiogenic target for the treatment of breast cancer angiogenesis and progression.

3.2 Introduction

Aggressive breast cancer cells exhibit stem cell-like properties that contribute to their plasticity and ability to metastasize (1,2). For instance, metastatic cancer cells over-express genes normally restricted to hESCs and the
expression of an embryonic signature is associated with poor prognosis (3,4). Nodal, an embryonic morphogen from the TGF-β superfamily, is a hESC-associated protein that becomes re-expressed during cancer progression (1,5-7). Nodal signals through the ALK4/7 and ActRIIB receptor complex, and its signalling is enhanced by Cripto-1 co-receptor. Receptor activation leads to phosphorylation and nuclear translocation of SMAD2/3. Recent studies demonstrate that Nodal promotes a dedifferentiated phenotype in melanoma, glioma and prostate cancer, and that it increases cancer cell invasion and tumourigenicity in these cancer types (5,7,8). Nodal expression is also correlated with breast cancer progression, such that Nodal is absent in normal breast tissues, yet is aberrantly expressed in invasive breast cancer lesions (6). The functional consequences of Nodal in breast cancer remain elusive.

Recent studies have linked the acquisition of stem cell markers to the enhancement of classical hallmarks of cancer, such as self-sufficiency in growth signals and the acquisition of invasive phenotypes, as these phenotypes tend to be key characteristics of stem cell functionality. Angiogenesis is another hallmark of cancer that has recently been correlated with the acquisition of stem cell markers. For example, CD105+ human renal carcinoma cells, expressing Nestin, Nanog, and Oct3/4, have been shown to shed microvesicles that contain mRNAs for pro-angiogenic growth factors (including, but not limited to, VEGF and FGF) to facilitate tumour angiogenesis (9). Stem cell-associated phenotypes have also been linked to angiogenic potential in gliomas. A mesenchymal-like subclass of high-grade gliomas has been shown to exhibit elevated expression of
pro-angiogenic factors including VEGF and PECAM, and a recent study revealed that glioma stem cell-like populations promote vascularization via a combinatorial mechanism involving the secretion of VEGF and Stromal-Derived Factor-1 (SDF-1) (10,11). Lastly, one study has reported that Nodal regulates VEGF expression and is correlated with vessel density in gliomas (12). However, this latter study did not directly assess the role of Nodal in the regulation of angiogenesis or vasculogenesis.

Here, we demonstrate that Nodal is a potential target for the treatment of breast cancer vascularization. We found that Nodal protein is positively associated with a high microvascular density in human breast cancer tissues, and that it causes vascular recruitment in vivo. In vitro, we found that Nodal increases the ability of breast cancer cells to promote tube formation by endothelial cells, and that it regulates the expression of VEGF and other pro-angiogenic factors in these breast cancer cells. Finally, using inducible shRNA technology, we determined that Nodal can be targeted in established tumours to decrease vascular density and induce necrosis. Given that Nodal is not present in non-lactating normal adult breast tissue, it may be useful as a therapeutic target against breast cancer angiogenesis.

3.3 Methods
3.3.1 Ethics Statement
Human archival tissue was obtained from the Ontario Institute for Cancer Research (OICR), Ontario Tumour Bank. All experiments involving animals were
approved by the Animal Use Subcommittee at the University of Western Ontario, Protocol No. 2008-101 (Appendix 2).

### 3.3.2 Immunohistochemistry (IHC) of human tissue

Formalin-fixed, paraffin-embedded archival tissue with clinical and pathological information was obtained from breast cancer patients (OICR). In total, ninety tumour sections, predominately reflecting invasive disease, were procured. Following deparaffinization in xylene, ethanol degradation, antigen retrieval with citrate buffer, and peroxidase and serum-free protein blocks, Nodal or CD31 specific antibodies were applied (Table 3.1). To verify the specificity of Nodal staining, a monoclonal versus a polyclonal antibody were compared (Figure 3.1). In confirmation of previous studies, these antibodies stained the same cell populations, and revealed comparable staining patterns (13). We proceeded with the monoclonal antibody, as it generated less background staining, and it has been shown to detect human Nodal in breast cancer, melanoma, and endometrial cancers in several previously published reports (5,6,14). Slides were rinsed in TBS-T, and treated with Envison+ HRP anti-mouse IgG (Dako). Color was produced with DAB substrate and counterstained with Mayer’s haematoxylin. Samples were dehydrated in reagent grade alcohol and cover slipped with permanent mounting medium. Isotype-matched mouse IgG negative controls were used at the same concentration as Nodal and CD31 antibodies.
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<td>1:500 (IHC)</td>
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<td>Polyclonal rabbit anti-PDGF</td>
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Table 3.1 Antibodies for Western Blot (WB), Immunofluorescence (IF) and Immunohistochemical (IHC) Analyses.
Figure 3.1 AbCAM Nodal antibody stains the same cells as R&D Nodal antibody, but with less non-specific background staining. Immunohistochemical staining (brown) of Nodal in serial sections of human breast cancer tissue specimens. Three examples of patients with low and high levels of Nodal expression are displayed, and 2 different antibodies are compared: A mouse anti-human Nodal monoclonal antibody (mAb) from AbCAM, and a goat anti-mouse Nodal polyclonal antibody (pAb) from R&D. The R&D antibody has been used successfully in several publications, and has been shown to detect human Nodal in breast cancer, melanoma and endometrial cancers (see Methods). The AbCAM mAb shows a similar staining pattern as the previously characterized antibody, but with less background staining. Hence, the AbCAM mAb was chosen for our experiments. Bar equals 100 mm.
3.3.3 Evaluation of Nodal IHC and tumour vascular density in breast cancer sections

Nodal IHC was evaluated under light microscopy. Cytoplasmic staining was seen in all cases. Cases were blindly and independently scored by two pathologists (S.J.D. and J.M.) to derive a total Allred score (15). Total Allred score is the sum of proportion score (percentage of cells stained: 0% = 0, <1% = 1, 1-10% = 2, 10-33% = 3, 34-66% = 4 and 67% = 5) and intensity score (No staining = 0, Weak = 1, Moderate = 2, Strong = 3). Values were dichotomized using a total Allred score of 7 and 8 as high expression, and ≤6 as low expression.

Vascular density was blindly and independently scored by two pathologists (S.J.D. and J.M.) based on CD31 IHC staining, following the recommendations from the International Consensus on Evaluation of Angiogenesis in Solid Human Tumours (16). Briefly, three “hotspots” in the stromal component of each tissue section were selected, the number of vessels in each “hotspot” was counted at 200x magnification, and a final score was expressed as the mean vessel density per section. Data was dichotomized using the average number of vessels among all samples as the cut-off between high and low vascular density values. All scoring values obtained by S.J.D. and J.M. had a Pearson Correlation Coefficient (rp) of 0.73 or higher. 83 out of 90 cases had both Nodal IHC and CD31 vascular density scores, and were used for statistical analyses.

For clinical characteristics of these 83 patients, see Table 3.2. The Allred scores for Nodal were used to assess correlations between Nodal, ER status, PR status, HER-2 status and tumour grade. For these correlation analyses, positive
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Table 3.2 Characteristics of the breast cancer patient tissue cohort.
ER/PR/HER-2 staining was assigned a value of 1 and negative staining was assigned a value of 0. The disease grade was assigned a value of 1, 2, or 3. Assessment of correlations was performed using Pearson Correlation and a 2-sided t-test.

3.3.4 Cell culture and transfection

Multiple cell types (both endothelial and breast cancer), treatments (drugs and recombinant proteins), and constructs (shRNA and expression vectors) were used throughout this study:

**Endothelial cells:** Two endothelial cell lines were used, human umbilical vein endothelial cells (HUVECs; ATCC), and primary human adult microvascular endothelial cells (HMVECs; Invitrogen). We used both adult and umbilical sources to account for possible functional differences in response to Nodal. Cells were maintained with attachment factor, and Medium 131 + Microvascular Growth Supplement as per manufacturer suggestions (Invitrogen).

**Breast cancer cells:** We used two highly aggressive breast cancer cell lines that express high endogenous Nodal for loss-of-function studies (MDA-MB-231 and MDA-MB-468), and one poorly aggressive breast cancer cell line that expresses low endogenous Nodal for gain-of-function studies (T47D) (Figure 2.1A). All breast cancer cell lines were obtained from and validated by ATCC, and were maintained as per instructions. Multiple constructs were used to knockdown or induce Nodal signalling in each of the cell lines used:

**Using shRNA or SB431542 for Nodal loss-of-function experiments:** Nodal knockdown was achieved in MDA-MB-231 cells with a pGIPZ lentiviral
shRNAmir against Nodal’s 3rd exon (Clone: V2LHS 155453, Open Biosystems), or with a pTRIPZ lentiviral shRNAmir against Nodal’s 3rd exon (Clone: V2THS 155453) with Tet-On /Tet-Off inducibility (Open Biosystems). Additionally, Nodal knockdown was achieved in MDA-MB-468 cells with a HuSH-29mer shRNA against Nodal’s 3rd exon (Id: GI311711) (Origene). Scrambled Control and anti-Nodal shRNAs were transfected using Arrest-In (Open Biosystems) or Lipofectamine (Invitrogen) as per manufacturer instructions, and cells were stably selected using Puromycin (200-450 ng/mL). For all knockdown systems, shRNAs targeting at least 4 regions in the Nodal gene were tested for their ability to knockdown Nodal protein expression. For each vector type, the shRNA achieving the best knockdown was chosen (as specified above). To inhibit Nodal signalling, the ALK4/5/7 inhibitor SB431542 (Sigma) was used at a concentration of up to 10 M. We chose to use 10 M of SB431542 in our experiment, since this dose consistently had the greatest inhibitory effect on SMAD2 phosphorylation, and has been used in previously published reports on hESCs to block Nodal signalling (17,18).

**Using expression vectors or recombinant protein for Nodal gain-of-function experiments:** For Nodal gain-of-function experiments, a Nodal expression vector was made with pcDNA 3.3-TOPO cloning kit, and an empty pcDNA3.3 vector was used as a control. Vectors were sequenced and validated, transfected into T47D cells using Lipofectamine (Invitrogen) as per manufacturer instructions, and stably selected with Geneticin (G418; 800 ng/mL). Additionally, for proof-of-principle, 50-100 ng/mL recombinant human Nodal (mature species)
was used to treat cells and activate phospho (P)-SMAD2 signalling (R D systems). We chose to test this concentration range since previous studies have shown that at least 50 ng/mL of Nodal is required for maintenance of pluripotency in hESCs (17,19). We chose to use 100 ng/mL of recombinant human Nodal (rhNodal) for our experiments (rather than 50 ng/mL), since we found there was substantial lot-to-lot variance, and 100 ng/mL most consistently induced phosphorylation of SMAD2. Furthermore, 100 ng/mL of rhNodal has been used effectively in multiple published studies related to Nodal in cancer (20,21).

3.3.5 RNA extraction and real time RT-PCR

Total RNA was isolated from cells and tumour tissue using the Perfect Pure RNA cultured cell kit (5 Prime) and genomic DNA was degraded using DNase. Reverse transcription was performed using 2 g RNA and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using TaqMan gene expression human primer/probe sets (Table 3.3). Gene expression was normalized to the endogenous control genes HPRT1 or RPLPO.

3.3.6 Western blotting

Protein lysates were prepared and quantified as previously described (6). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, and resolved proteins were transferred onto Immobilon-P membranes (Millipore Corp.). Membranes were incubated with primary antibody (Table 3.1) and the appropriate horseradish peroxidase-
Table 3.3 Primer/Probe information for real-time PCR (Applied Biosystems)

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe ID</th>
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</thead>
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<tr>
<td>PDGF-B</td>
<td>Hs00234042_m1</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Hs00173626_m1</td>
</tr>
<tr>
<td>RPLPO</td>
<td>4333761-0707014</td>
</tr>
<tr>
<td>HPRT1</td>
<td>4333768-0904021</td>
</tr>
</tbody>
</table>
conjugated secondary antibody. Enzyme activity was detected by enhanced chemiluminescence (Super Signal; Pierce). Images were obtained using film.

In accordance with previous studies (5,6,20,22), three banding locations were detected for Nodal: Pro-Nodal at 39 kDa, fully processed Nodal at 50 kDa, and mature Nodal at 15 kDa. The 50 kDa species is highly variable due to differences in post-translational modifications and protein lysate handling. The 15 kDa band is typically in low abundance in both cell lysates and conditioned media due to low protein stability (22); however, it is slightly easier to pick up in condensed conditioned media. For consistency and accuracy, we used the 39 kDa band to assess Nodal expression in lysates (as we have previously shown that it is proportional to 15 kDa mature Nodal in both lysate and conditioned media (20)), and the 15 kDa band to assess Nodal levels in conditioned media.

3.3.7 In vitro functional assays

Functional assays were performed on endothelial cell lines (HUVECs and HMVECs) in response to conditioned media from either (1) MDA-MB-231 cells transfected with a scramble control shRNA or a Nodal-targeted shRNA (pGIPZ constructs as specified in Section 3.3.4), or (2) T47D cells transfected with an empty control vector or a Nodal expression vector.

**Tube formation:** Endothelial cell Medium 131 supplemented with 0.5% BSA was conditioned by cancer cells for 24 hours. Endothelial cells were seeded on Matrigel and treated with conditioned media. In some cases, rhNodal (100 ng/mL; R&D), VEGF (20 ng/mL; Invitrogen), and/or PDGF (20 ng/mL; Invitrogen)
were added to the conditioned medium after it was collected, during the course of the assay. 20 ng/mL of VEGF and PDGF was used because it is the minimum concentration consistently reported to stimulate endothelial cells in vitro (23,24), and it is much lower than concentrations used for most in vivo positive controls (for example, refer to DIVAA manufacturer protocol). Tube formation was quantified by counting the number of circular structures that formed from complete branching per field of view (20x magnification). A sum of 10 fields of view was used for each treatment replicate.

**Migration:** Endothelial cells (50,000 cells) were seeded into a Transwell insert, and treated with conditioned media from cancer cells. Migration (24 hrs) was quantified by staining nuclei with DAPI and counting cells. In some cases, rhNodal, VEGF, and/or PDGF were added to the conditioned medium after it was collected, during the course of the assay. An average of 10 fields of view (20x magnification) was used for each treatment replicate.

**Viability:** Viability of endothelial cells in response to conditioned media was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit as per manufacturer's instructions (Invitrogen).

### 3.3.8 SearchLight protein array

In some cases (as indicated) VEGF and PDGF protein levels were quantified by an Aushon SearchLight Chemiluminescent Angiogenesis Arrays (84694; Aushon Biosystems, Billerica, MA) as per manufacturer instructions.
3.3.9 Directed in vivo angiogenesis assay (DIVAA)

A DIVAA kit was used for the in vivo angiogenesis assays (Trevigen, Gaithersburg, MD) (25,26). Angioreactor tubes were filled with Matrigel (negative control), Matrigel + cancer cells, or Matrigel + cancer cells + growth factors (for rescue analyses). As in our in vitro assays, rhNodal was added at a concentration of 100 ng/mL, and VEGF was added at a concentration of 20 ng/mL. Angioreactors were inserted subcutaneously into nude mice. After 10 days, mice were sacrificed and angioreactors were removed. Blood vessels that invaded the angioreactors were imaged under a microscope, and subsequently transferred into a 1.5 mL tube. Endothelial cells were incubated with FITC-Lectin overnight (provided in the DIVAA kit), and fluorescence was quantified using a plate reader.

3.3.10 Chick chorioallantoic membrane (CAM) assays

To assess angiogenesis, one collagen-enmeshed grid containing 2 X 10^6 cells was placed on each CAM, and newly formed blood vessels that appeared in the upper grids of the collagen onplants after 3 days were scored and quantified as previously described (27). To assess tumour formation, 100,000 cells were placed directly onto the CAM, and were left for 5 days prior to confocal imaging. Tumour cells were visualized with GFP expressed in the bicistronic pGIPZ shRNA constructs. In both cases, recombinant VEGF was added at a concentration of 20 ng/mL for rescue analysis.
3.3.11 In vivo inducible shRNA tumour assay

MDA-MB-231 cells were transfected with a doxycyclin (Dox)-inducible control or Nodal-targeted shRNA. Both constructs express RFP upon induction by Dox. 500,000 cells in 100 L RPMI + Matrigel (1:1) were injected into the right flank of 6-8 week old female Nude mice (Crl:NU-\textit{Foxn1}\textsuperscript{nu}; Charles River). Dox was administered 2 weeks following onset of palpable tumour growth via diet (0.625 g doxycycline hyclate per 1 kg chow). This diet is optimized to deliver Dox at 2-3 mg/day, given that each mouse eats approximately 4-5 g/day (Harlan Laboratories). Excised tumours were cut in half: one half was subject to formaldehyde-fixation for H E and analysis of tumour necrosis, and the other half frozen and cryosectioned for IF and analysis of tumour hypoxia. Following cryosectioning, remaining tumour tissue was used for RNA extraction and PCR analysis.

**Immunofluorescence:** Immunofluorescence was conducted using anti-CD31 and anti-Nodal antibodies (Table 3.1) with the appropriate fluorochrome-conjugated secondary antibodies. DAPI was used to stain nuclei and IgG isotype controls were performed. For each tumour section, CD31 was quantified by taking 5 random images of vascular hotspots around the tumour periphery (tumour cores were too necrotic to include), and then counting the number of vessels in each field of view across a 25-box grid overlay using ImageJ software. For each tumour, 3 serial sections were quantified by this method, and averaged to yield one value for CD31 expression per mouse replicate.
**Analysis of VEGF expression:** After cryosectioning, remaining frozen tumour tissue was subject to RNA extraction as per manufacturer’s protocol (described in Section 3.3.5). VEGF mRNA expression was subsequently analyzed by real time RT-PCR, as described above.

**Analysis of tumour necrosis:** Three tumour sections spaced evenly throughout each tissue block were stained with H E. Each section was imaged such that the entire section was visible in one picture. Photoshop software was used to outline and quantify total tumour area, and area of necrosis. Necrosis was calculated as a percentage of the total tumour area.

**Analysis of tumour hypoxia:** Animals were injected with hypoxyprobe-1 (60 mg/kg body weight) prior to sacrificing. Excised tumours were frozen and IHC was performed as described above, with a few modifications as per manufacturer instructions. Acetone was used for fixation. Endogenous immunoglobulins in mouse tissue were blocked with the mouse-on-mouse (M.O.M. ) basic kit (Vector Laboratories). Sections were incubated with anti-hypoxyprobe-1 antibody (HPI, Inc) (Table 3.1), and a biotinylated anti-mouse IgG (H+L) secondary antibody (M.O.M. kit, Vector Laboratories). Vectastain ABC kit (Vector Laboratories) was used to detect enzyme activity, DAB substrate (Dako) was used for color development, and haematoxylin was used to counterstain.

**3.3.12 Statistical analyses**

Statistical analyses of multiple comparisons for parametric data were performed using a one-way ANOVA followed by a Tukey Kramer Comparisons Post-Hoc test. An ANOVA on Ranks followed by the Mann-Whitney rank-sum test was
used for non-parametric data. When only two items were compared, a student’s t-test was used. Parametric data were expressed as mean S.E.M. for replicate values, and non-parametric data were expressed as median interquartile range. All statistical tests were two-sided and data comparisons for all experiments were considered statistically significant at \( p<0.05 \). Statistics were performed using SigmaStat (Dundas Software) in consultation with the biostatistical support unit at the University of Western Ontario.

### 3.4 Results

#### 3.4.1 Nodal is correlated with CD31 in human breast cancer samples

Angiogenesis is a rate-limiting step in the metastatic cascade and high microvascular density is correlated with a poor clinical outcome (28,29). Given recent evidence linking tumour angiogenesis to stem cell markers, we sought to determine if Nodal expression correlates with microvascular density in human breast cancers. Immunohistochemical analysis of Nodal and CD31 (to demarcate endothelial cells) in a cohort of breast cancer patients was examined in serial sections (29). Breast cancer tissue from 90 patients was procured from the primary tumour site; 89 via surgical resection and 1 via excisional biopsy. The average age of the patients was 61 years (ranging from 27 to 90) with a SD of 16.5 years. The majority of the cases were invasive disease, diagnosed as being Grade 3 (47%) or Grade 2 (37.4%). Of the 90 samples, 83 could be scored for both Nodal and vascular density, and were used for statistical analyses. Clinical characteristics of these patients are listed in Table 3.2. IHC for both Nodal and CD31 was heterogeneous, varying between individuals and within sections in
extent, intensity and localization. In these lesions, Nodal was generally confined
to epithelial-like tumour cells (Figure 3.1, Figure 3.2A). Nodal staining was not
correlated to breast cancer Grade (p=0.140), or to Estrogen Receptor (ER)
(p=0.682), Progesterone Receptor (PR) (p=0.801) or HER2 (p=0.589) status.
However, high levels of Nodal were associated with a high vascular density
(n=83; rp=0.3, p=0.0076), suggesting a clinical association between the
acquisition of Nodal expression and vascular recruitment (Figure 3.2A,B).

3.4.2 Nodal promotes endothelial cell tube formation in vitro

To explore whether Nodal regulates breast cancer angiogenesis, we first
tested the effects of Nodal on endothelial cell function. Specifically, we performed
in vitro tube formation and migration assays using human umbilical vein
endothelial cells (HUVECs), or primary human microvascular endothelial cell
(HMVECs) treated with conditioned media from stably transfected breast cancer
cells. For Nodal loss-of-function experiments, we used MDA-MB-231 cells, which
express high levels of Nodal, and for Nodal gain-of-function experiments, we
used T47D cells, which express relatively low levels of Nodal (Figure 2.1A) (6).
We found that conditioned media from MDA-MB-231 cells transfected with a
Nodal-targeted shRNA (231+shNodal) significantly reduced endothelial
branching (HUVEC n=3, P < 0.001; HMVEC n=3, P < 0.001) and migration (HUVEC
n=4, P < 0.001; HMVEC n=3, P < 0.001) of both HUVECs and HMVECs compared
to conditioned media from MDA-MB-231 cells transfected with a Control shRNA
(231+shControl) (Figure 3.3A-D, Figure 3.4A-C). This effect was not due to
altered cell viability or proliferation (Figure 3.3E-F, Figure 3.4D). Similarly, in
A

Low Nodal Patient

High Nodal Patient

H&E

Nodal

CD31

IgG Control

B

<table>
<thead>
<tr>
<th>Vascular Density</th>
<th>Low</th>
<th>High</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>32</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>15</td>
<td>22</td>
<td>0.008</td>
</tr>
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</table>
Figure 3.2 High Nodal expression is associated with vascular hotspots in breast cancer lesions. (A) Immunohistochemical staining (brown) of Nodal and CD31 in serial sections of human breast cancer tissue specimens. Examples of a patient with a low level of Nodal expression and another patient with a high level of Nodal expression are displayed. Sections stained with H&E or incubated with an isotype control are also included. Bar equals 50 μm. (B) Entire sections of each breast cancer case were analyzed to derive an Allred Score for Nodal expression. A total Allred score of 7 and 8 represented high Nodal expression and a score of 6 and below represented low Nodal expression. In serial sections, vascular density was scored based on CD31 staining. Following the recommendations from the International Consensus on Evaluation of Angiogenesis in Solid Human Tumors, three “hotspots” in the stromal component were selected, the number of vessels in each “hotspot” was counted and a final score was expressed as the mean vessel density. Data was dichotomized using the average number of vessels among all the samples as the cut-off between high and low vascular density values. High Nodal expression was found to be associated with a high vascular density in human breast cancer tissue (n=83 patients, rp=0.3, P=0.0076).
**A**

**Nodal (CM)**

---

**B**

**Loss of Function: HUVEC**

**C**

**HUVEC Tube Formation**

- 0.5% BSA
- shControl
- shNodal
- shNodal + rhNodal

**D**

**HUVEC Migration**

- 0.5% BSA
- shControl
- shNodal
- shNodal + rhNodal

**E**

**HUVEC Viability (Rel. to BSA control)**

- 0.5% BSA
- shControl
- shNodal
- shNodal + rhNodal

**F**

**Number of HUVECs (x10^6)**

- 0 ng/mL rhNodal
- 100 ng/mL rhNodal
Figure 3.3 Nodal inhibition in MDA-MB-231 cells reduces HUVEC tube formation and migration in vitro. (A) Western blot analysis of Nodal protein in conditioned medium of 231+shNodal cells or 231+shControl cells. The 15 kDa cleaved and secreted Nodal band is presented. (B) Representative images of HUVEC Matrigel tube formation assays treated with 0.5% BSA (negative control), 231+shControl-conditioned media, 231+shNodal-conditioned media, or 231+shNodal-conditioned media plus the addition of recombinant human Nodal (rhNodal). Bar equals 100 μm. (C) Quantified HUVEC tube formation corresponding to (B). Nodal knockdown caused a significant reduction in tube formation compared to 231+shControl, and this effect was not rescued by addition of rhNodal to conditioned media. Bars represent mean number of branch points S.E.M. (n=3, P<0.001). (D) Transwell assay testing HUVEC cellular migration in response to treatments from (B) and (C). Nodal knockdown caused a significant reduction in HUVEC migration compared to 231+shControl, and this effect was rescued by addition of rhNodal to conditioned media. Bars represent mean number of migrated cells per field of view (FOV) S.E.M. (n=4, P<0.001). (E) Viability assay testing the effects of treatment conditions from (B-D) on HUVECs. Viability was not compromised in response to conditioned media. Bars represent fold change in fluorescence S.E.M. relative to 0.5% BSA control (n≥4, P=0.2). (F) HUVEC cell proliferation assay over 4 days in response to treatment with 100 ng/mL rhNodal. No significant difference was observed for any of the time points (n=3, p 0.05). For all graphs, different superscripted letters indicate a statistical difference as specified.
A. Loss of Function: HMVEC

- Ve Control (0.5% BSA)
- 231+shControl CM
- 231+shNodal CM -ve Control (0.5% BSA)
- 231+shNodal CM
- +rhNodal

B. HMVEC Tube Formation

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<tr>
<th>Condition</th>
<th>No. of branch points/10x FOV</th>
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<td>0.5% BSA</td>
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<tr>
<td>shControl</td>
<td>160</td>
</tr>
<tr>
<td>shNodal</td>
<td>120</td>
</tr>
<tr>
<td>shNodal + rhNodal</td>
<td>80</td>
</tr>
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</table>

C. HMVEC Migration

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<tr>
<td>shControl</td>
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<tr>
<td>shNodal</td>
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<tr>
<td>shNodal + rhNodal</td>
<td>10</td>
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</table>

D. HMVEC Viability

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<td>shControl</td>
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<td>shNodal</td>
<td>0.85</td>
</tr>
<tr>
<td>shNodal + rhNodal</td>
<td>0.80</td>
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</table>
Figure 3.4 Nodal inhibition in MDA-MB-231 cells reduces HMVEC tube formation and migration in vitro. (A) Representative images of primary HMVEC Matrigel tube formation assays treated with 0.5% BSA (negative control), 231+shControl-conditioned media, 231+shNodal-conditioned media, or 231+shNodal-conditioned media plus the addition of rhNodal. Bar equals 50 μm. (B) Quantified HMVEC tube formation corresponding to (A). Nodal knockdown caused a significant reduction in tube formation compared to 231+shControl, and this effect was not rescued by addition of rhNodal to conditioned media. Bars represent mean number of branch points ± S.E.M. (n=3, P<0.001). (C) Transwell assay testing HMVEC cellular migration in response to treatments from (A) and (B). Nodal knockdown caused a significant reduction in HMVEC migration compared to 231+shControl, and this effect was not rescued by addition of rhNodal to conditioned media. Bars represent mean number of migrated cells per field of view (FOV) ± S.E.M. (n=4, P<0.001). (D) Viability assay testing the effects of treatment conditions from (A-C) on HMVECs. Viability was not compromised in response to conditioned media. Bars represent fold change in fluorescence ± S.E.M. relative to 0.5% BSA control (n=6, P>0.05).
complementary Nodal gain-of-function experiments, we treated HUVECs with conditioned media from T47D cells transfected with an empty vector (T47D+EV) or a Nodal expression construct (T47D+Nodal) (Figure 3.5A). We found that tube formation (n=3, P<0.01) and migration (n=4, P<0.05) were significantly increased in response to conditioned media from T47D+Nodal cells, compared to T47D+EV cells (Figure 3.5B-D). This effect was not due to a change in HUVEC viability (Figure 3.5E).

In order to determine if Nodal was acting directly on the endothelial cells, or indirectly by regulating the expression of angiogenic factors in the cancer cells, we added recombinant human Nodal (rhNodal) to the collected conditioned media during the course of the tube formation assays. If Nodal was acting directly on the endothelial cells, rhNodal would restore endothelial cell function in 231+shNodal-conditioned media. Although migration of HUVECs was rescued by rhNodal treatment, migration of HMVECs and tube formation for both endothelial cell types were not affected (Figure 3.3B-E, Figure 3.4A-D). This suggests that Nodal acts on some endothelial cells directly, but also that Nodal can promote angiogenesis indirectly, perhaps by up-regulating the secretion of pro-angiogenic proteins by the cancer cells.

In support of this concept, using a SearchLight array and Western blot analysis, we found that Nodal inhibition with shRNA caused a significant reduction in Platelet Derived Growth Factor (PDGF) (n=3, p=0.018) and VEGF (n=3, p=0.007) protein expression (Figure 3.6A-D). Furthermore, addition of rhNodal (100 ng/mL) to 231+shNodal cells rescued VEGF expression as
Figure 3.5 Nodal overexpression in T47D cells promotes HUVEC tube formation and migration in vitro. (A) Western blot validation of elevated Nodal expression in T47D+Nodal conditioned media versus T47D+EV stably transfected cells. The ~15 kDa mature Nodal band is shown. (B) Representative images of HUVEC Matrigel tube formation assays treated with 0.5% BSA (negative control), 20 ng/mL VEGF-A (positive control), T47D+EV-conditioned media, or T47D+Nodal-conditioned media. Bar equals 100 mm. (C) Quantified tube formation corresponding to images in (B). HUVECs formed tubes more efficiently when treated with T47D+Nodal-conditioned media compared to T47D+EV-conditioned media. Bars represent mean number of branch points ± S.E.M. (n=3, P<0.01). (D) Transwell assay testing HUVEC cellular migration in response to treatments from (B) and (C). HUVEC migration was significantly increased in response to T47D+Nodal-conditioned media compared to T47D+EV-conditioned media (n=4, P<0.05). Bars represent mean number of migrated cells per field of view (FOV) ± S.E.M. (E) Viability assay testing the effects of treatment conditions from (B-D) on HUVECs. Viability was not compromised in response to conditioned media. Bars represent fold change in fluorescence ± S.E.M. relative to 0.5% BSA control (n=3, P=0.48). For all graphs, different superscripted letters indicate a statistical difference as specified.
Figure 3.6 Nodal inhibition in MDA-MB-231 and MDA-MB-468 cells reduces expression and secretion of pro-angiogenic factors. (A) Western blot validating Nodal knockdown in 231+shNodal versus 231+shControl cells, concomitant with a reduction in phosphorylated-SMAD2 (indicative of decreased canonical Nodal signalling). The pro-Nodal (~39 kDa) band is detected and SMAD2/3 and Actin are used as loading controls. (B) Western blot demonstrating that VEGF protein in 231+shNodal lysate is lower than in 231+shControl lysate, but that VEGF expression is rescued by the addition of 100 ng/mL rhNodal. (C) PDGF protein expression (PDGF n=3, p=0.018), and (D) VEGF protein expression (VEGF n=3, p=0.007) were reduced in 231+shNodal cells compared to 231+shControl cells as measured with a protein array. Bars represent mean protein expression ± S.E.M. (E) SearchLight protein quantification of PDGF expression in conditioned media (n=3, p=0.029) or (F) cell lysate (n=3, p<0.001) from 468+shControl versus 468+shNodal cells. (G) SearchLight protein quantification of VEGF expression in conditioned media (n=3, p<0.001) or (H) cell lysate (n=3, p<0.001) from 468+shControl versus 468+shNodal cells. (I) Western blot demonstrating reduced VEGF expression in 468+shNodal cells compared to 468+shControl cells. For all graphs, asterisks (*) indicate a significant difference compared to controls as specified.
measured by Western blot (Figure 3.6B). To validate these observations, we measured VEGF and PDGF protein in response to Nodal knockdown in MDA-MB-468 cells. We found that Nodal inhibition caused decreased expression of PDGF in conditioned media (n=3, p=0.029) and cell lysate (n=3, p<0.001) (Figure 3.6E,F), and decreased expression of VEGF in conditioned media (n=3, p<0.001) and cell lysate (n=3, p<0.001) (Figure 3.6G-I). In complementary gain-of-function experiments, treatment of T47D or MCF-7 cells with increasing concentrations of rhNodal (0, 50, or 100 ng/mL) resulted in an increase in VEGF and PDGF protein expression measured by Western blot (Figure 3.7A,B). Furthermore, treatment of T47D cells with rhNodal caused a significant increase in VEGF and PDGF mRNA expression (n=3, P<0.05) (Figure 3.7C). Similarly, using a SearchLight protein array, we found that T47D+Nodal cells displayed a significant increase in PDGF protein expression in conditioned media (n=3, p=0.045) and cell lysate (n=4, p=0.029) (Figure 3.7D,E), and a significant increased in VEGF protein expression in cell lysate (n=3, p=0.029) compared to T47D+EV cells (Figure 3.7F-H). Lastly, we found that activation of ALK4/7 is required for VEGF and PDGF regulation, since treatment of T47D cells with SB431542 inhibits rhNodal-induced up-regulation of these proteins (Figure 3.7I).

Given that Nodal regulates pro-angiogenic proteins in breast cancer cells, we next investigated whether we could rescue the effects of 231+shNodal-conditioned media on endothelial cells with recombinant VEGF and/or PDGF. We added these proteins to the conditioned media after it was collected from the cancer cells (i.e. during the incubation on endothelial cells). Unlike the addition of
Figure 3.7 Nodal overexpression in T47D cells increases expression and secretion of pro-angiogenic factors. (A) Western blot validating dose-dependent induction of Nodal signaling via phospho-SMAD2 in T47D cells in response to vehicle or rhNodal (50 or 100 ng/mL) after 24 hrs. Actin was used as a loading control. (B) Western blot analysis showing a dose-dependent increase in VEGF and PDGF in T47D cells or MCF-7 cells treated with increasing concentrations of rhNodal (0, 50, or 100 ng/mL). Actin is used as a loading control. (C) Real-time RT-PCR analysis of VEGF and PDGF expression in T47D cells treated for 24 hours with either vehicle or rhNodal (50 ng/mL, 100 ng/mL). VEGF and PDGF mRNA expression were significantly elevated in T47D cells treated with 50 or 100 ng/mL of rhNodal. Expression levels are normalized to HPRT1. Bars represent mean gene expression ± S.E.M. relative to 0 ng/mL rhNodal control (n=3, P<0.05 for each). (D) SearchLight protein quantification of PDGF expression in conditioned media (n=3, p=0.045) or (E) cell lysate (n=3, p=0.029) from T47D+EV versus T47D+Nodal cells. (F) SearchLight protein quantification of VEGF expression in conditioned media (n=3, p>0.05) or (G) cell lysate (n=3, p=0.029) from T47D+EV versus T47D+Nodal cells. (H) Western blot demonstrating elevated VEGF expression in T47D+Nodal cells compared to T47D+EV cells. Actin is used as a loading control. (I) Western blot demonstrating that VEGF and PDGF are up-regulated in response to rhNodal treatment (100 ng/mL) in T47D cells, but that this is mitigated with the addition of SB431542 (10 μM). Actin is used as a loading control. For all graphs, asterisks (*) indicate a significant difference compared to controls as specified.
rhNodal, which did *not* rescue angiogenic phenotypes compared to controls, we found that addition of VEGF and/or PDGF to 231+shNodal conditioned media during the tube formation and migration assays *did* restore both phenotypes in HUVEC and HMVEC cultures (**Figure 3.8A-C, Figure 3.9A-C**). This was not due to a significant change in viability (**Figure 3.8D, Figure 3.9D**).

### 3.4.3 Nodal promotes vascular recruitment in vivo

To test the significance of our *in vitro* results we employed a directed *in vivo* angiogenesis assay (DIVAA). Stably transfected cancer cells were seeded into silicone matrix-containing angioreactors, which were implanted subcutaneously into nude mice, and endothelial cells that invaded the angioreactors were quantified using a FITC-lectin-based assay (25). We determined that 468+shControl cells (n=7, p=0.007) and 231+shControl cells (n=10, p=0.003) efficiently recruit vasculature within 10 days, and that this ability is mitigated when Nodal is knocked down (**Figure 3.10A-D**). Moreover, this *in vivo* effect of Nodal knockdown on angiogenesis was fully rescued in 468+shNodal cells by the addition of VEGF (**Figure 3.10C,D**). In contrast to our *in vitro* studies, we were able to partially recover the effects of Nodal knockdown with the addition of rhNodal (**Figure 3.10C,D**). This may have been because the cancer cells in the implants responded to the rhNodal by re-expressing VEGF and PDGF. Alternatively, Nodal may affect other pro-angiogenic cell types *in vivo*, such as hematopoietic stem cells, not accounted for in our endothelial cell assays. In accordance with our *in vitro* studies, T47D+Nodal transfected cells induced significantly higher vessel recruitment compared to controls (n=10,
A

HUVEC Viability (Rel. to BSA control)

HUVEC Tube Formation (No. of branch points/10^20x FOV)

HUVEC Migration (Average Number of cells/20x FOV)

0.5% BSA shControl shNodal shNodal +VEGF shNodal +PDGF shNodal +VEGF +PDGF

B

0
10
20
30
40
50
60
70
80

HUVEC Tube Formation (No. of branch points/10^20x FOV)

C

0
10
20
30
40
50

HUVEC Migration (Average Number of cells/20x FOV)

D

0
0.4
0.8
1.2
1.6

HUVEC Viability (Rel. to BSA control)

shControl shNodal shNodal +VEGF shNodal +PDGF shNodal +VEGF +PDGF

231+shNodal CM 231+shControl CM -ve Control (0.5% BSA)
Figure 3.8 VEGF and PDGF rescue the effects of Nodal knockdown on HUVEC tube formation and migration. (A) Representative images of HUVEC Matrigel tube formation assays treated with 0.5% BSA (negative control), 231+shControl-conditioned media, 231+shNodal-conditioned media, or 231+shNodal-conditioned media plus the addition of VEGF and/or PDGF. Bar equals 100 mm. (B) Quantified HUVEC tube formation corresponding to (A). The effects of Nodal knockdown on tube formation are rescued by adding VEGF, or VEGF+PDGF to conditioned media following collection from cancer cells. Bars represent mean number of branch points ± S.E.M. (n=3, P<0.001). (C) Transwell assay testing HUVEC cellular migration in response to treatments from (A) and (B). The effects of Nodal knockdown on HUVEC migration are rescued by adding VEGF, PDGF, or VEGF+PDGF to conditioned media following collection from cancer cells. Bars represent mean number of migrated cells per field of view (FOV) ± S.E.M. (n=4, P<0.001). (D) Viability assay testing the effects of treatment conditions from (A-C) on HUVECs. Viability was not compromised in response to conditioned media. Bars represent fold change in fluorescence ± S.E.M. relative to 0.5% BSA control (n≥4, P=0.2). For all graphs, different superscripted letters indicate a statistical difference as specified.
A

HMVEC

B

HMVEC Tube Formation
(No. of branch points/10x FOV)

C

HMVEC Migration
(Average Number of cells/20x FOV)

D

HMVEC Viability
(Re. to BSA control)
Figure 3.9 VEGF and PDGF rescue the effects of Nodal knockdown on HMVEC tube formation and migration. (A) Representative images of primary HMVEC Matrigel tube formation assays treated with 0.5% BSA (negative control), 231+shControl-conditioned media, 231+shNodal-conditioned media, or 231+shNodal-conditioned media plus the addition of VEGF and/or PDGF. Bar equals 50 mm. (B) Quantified HMVEC tube formation corresponding to (A). The effects of Nodal knockdown on tube formation is rescued by adding VEGF, PDGF, or VEGF+PDGF to conditioned media following collection from cancer cells. Bars represent mean number of branch points ± S.E.M. (n=3, P<0.001). (C) Transwell assay testing HMVEC cellular migration in response to treatments from (A) and (B). The effects of Nodal knockdown on HMVEC migration is rescued by adding VEGF+PDGF to conditioned media following collection from cancer cells, and partially rescued by adding VEGF alone. Bars represent mean number of migrated cells per field of view (FOV) ± S.E.M. (n=4, P<0.001). (D) Viability assay testing the effects of treatment conditions from (A-C) on HMVECs. Viability was not compromised in response to conditioned media. Bars represent fold change in fluorescence ± S.E.M. relative to 0.5% BSA control (n=6, P>0.05). For all graphs, different superscripted letters indicate a statistical difference as specified.
Figure 3.10 Nodal promotes vascular recruitment in a Directed In Vivo Angiogenesis Assay (DIVAA). (A) 231+shNodal cells or 231+shControl cells were injected into silicon angioreactors and implanted subcutaneously into nude mice. After 10 days, vascular recruitment into the angioreactors was quantified using a FITC-lectin-based assay. Nodal knockdown caused a significant reduction in vascular recruitment compared to 231+shControl. Data are non-parametric and are presented as median ± interquartile range (n=10, P=0.003) (B) Images of representative angioreactors corresponding to (A) following excision from mice. (C) 468+shNodal cells or 468+shControl cells were injected into silicon angioreactors and implanted subcutaneously into nude mice for 10 days as described in (A). 468+shNodal cells had significantly reduced vascular recruitment compared to 468+shControl cells. This effect was partially rescued by the addition of rhNodal to angioreactors containing 468+shNodal cells, and was significantly rescued by the addition of VEGF. Recombinant VEGF in Matrigel (no cells) was included as a control. Data are parametric and are presented as mean relative fluorescent units ± S.E.M. (n=7, P<0.007). (D) Images of representative angioreactors corresponding to (C) following excision from mice. (E) T47D+Nodal or T47D+EV transfected cells were injected into silicon angioreactors and implanted subcutaneously into nude mice for 10 days as described in (A). T47D+Nodal cells had significantly increased vascular recruitment compared to T47D+EV cells (n=10, P<0.001). Bars represent mean relative fluorescent units ± S.E.M. (F) Images of representative angioreactors corresponding to (E) following excision from mice. For all graphs, different superscripted letters have a statistical difference.
P < 0.001) (Figure 3.10E,F). Collectively, these data confirm that Nodal promotes tumour angiogenesis in vivo.

As proof-of-principle of our DIVAA results, we used a Chick Chorioallantoic Membrane (CAM) assay as an additional method to assess the role of Nodal during vascularization in vivo. The CAM assay involves placing cells-of-interest on a collagen-enmeshed grid on the CAM and then scoring for newly formed blood vessels in the upper grids after 3 days (27). We found that 231+shControl cells were able to induce angiogenesis significantly better than 231+shNodal cells (n ≥ 37, P < 0.001) (Figure 3.11A). Furthermore, inclusion of recombinant VEGF with 231+shNodal cells on the CAM resulted in a rescue of the angiogenic phenotype (Figure 3.11A). Consistently, when we used the chick CAM to perform a tumour assay over 5 days, we found that 231+shNodal cells were unable to form tumours compared to controls by confocal analysis (Figure 3.11B). Furthermore, despite promoting vascularization, VEGF did not rescue 231+shNodal tumour formation (Fig. 311B), suggesting that Nodal may regulate multiple aspects of tumourigenesis in this model.

3.4.4 Inducible Nodal inhibition following tumour formation reduces vascularization

We next designed a clinically relevant model to evaluate the effects of targeting Nodal in established tumours. MDA-MB-231 cells transfected with a Doxycyclin-inducible Nodal shRNA (231+ishN) or Control shRNA (231+ishC) were injected with Matrigel into the flanks of nude mice. Dox treatment groups were given regular chow for the course of the experiment, whereas +Dox
Relative Angiogenesis
(Fold change relative to PBS control)

A

PBS   231+shControl   231+shNodal   231+shNodal +VEGF

B

231+shControl   231+shNodal   231+shNodal +VEGF
Figure 3.11 Nodal promotes vascular recruitment in a Chick Chorioallantoic Membrane (CAM) assay. (A) 231+shControl cells, 231+shNodal cells, or 231+shNodal cells +VEGF were inoculated onto chick CAMs with a collagen mesh grid. After 3 days, 231+shControl cells induced significantly more angiogenesis compared to 231+shNodal cells. This effect was rescued by the addition of VEGF to 231+shNodal cells. Bars represent mean angiogenesis relative to a PBS control S.E.M. (n≥37, P<0.001). (B) Confocal images of a 5-day tumor assay performed on chick CAMs. 231+shControl cells formed large tumors compared to 231+shNodal cells. Addition of VEGF to 231+shNodal cells did not rescue tumour formation. Vessels are shown in red, and cells are GFP-labelled (green). For all graphs, different superscripted letters have a statistical difference.
treatment groups were given chow containing Doxycycline during weeks 2-4 of the experiment (i.e. after the tumour had begun to form) (Figure 3.12A,B). We found that if we administered Dox after the tumour had already been established, we could avoid significant differences in tumour size between treatments over the course of the experiment, and thus minimize confounding effects of tumour growth on our analysis of angiogenesis. Of note, we found no significant difference in tumour size over time between treatments (n≥4, p=0.4) (Figure 3.12C). Following tumour excision, we performed immunofluorescence and noticed that the distribution of Nodal mirrored the distribution of CD31 across treatments, and that CD31 expression in 231+ishN +Dox tumours was often shallow and limited to the tumour periphery. Indeed, we found a 50% reduction in CD31 staining in 231+ishN +Dox tumours compared to all other treatments (n≥4, p=0.04) (Figure 3.13A,B). Interestingly, in cases where Nodal expression rebounded following Dox treatment in 231+ishN mice (a common limitation of shRNA technology), CD31 expression was similarly restored (Figure 3.13C). In accordance with these findings, we found that 231+ishN +Dox exhibited a significant reduction in VEGF expression compared to all other treatment groups (n≥3, p=0.003) (Figure 3.13D). Significant differences in PDGF expression were not, however, detected.

As a corollary to our CD31 and VEGF measurements, we wanted to evaluate whether inducible Nodal inhibition affected tumour necrosis. Using H&E analyses, we found that tumour necrosis was significantly higher and frequently extended closer to the tumour periphery in 231+ishN +Dox tumours compared to
A. Flank injection (500,000 cells)  
- 2 weeks: Administration of Doxycyclin diet  
- 4 weeks: Sacrifice mouse, Excise tumor  

% tumor flash freeze  
- Immunofluorescence: RFP validation CD31  
- Analysis of CD31 distribution

% tumor paraffin-embed  
- Immunohistochemistry H&E  
- Analysis of necrosis

B.  
kDa  
231+ishC  231+ishN  
39-  42-  
Nodal Actin  
- + - +  
Dox

C.  
231+ishC  231+ishN  
-Dox +Dox

D.  
Tumor Diameter (mm)  
Week 0 Week 2 Week 3 Week 4  
-shC +Dox  -shC -Dox  -shN -Dox  -shN +Dox
Figure 3.12 Experimental design for inducible shRNA tumour model in vivo. (A) Schematic representation of inducible shRNA tumor model. Briefly, 500,000 doxycyclin-inducible 231+ishC or 213+ishN stably transfected cells were injected with Matrigel into the flanks of nude mice. Mice were given normal chow for the course of the experiment (-Dox), or normal chow for 2 weeks followed by Doxycyclin-chow for 2 weeks (+Dox). After 4 weeks, tumors were excised and cryosectioned for immunofluorescence, or paraffin-embedded for H E. (B) Western blot analysis confirmed that the shRNA constructs were efficiently transfected and operated as expected in response to Doxycyclin in vitro. The 39 kDa Pro-Nodal band is depicted and Actin is used as a loading control. (C) Confocal images of 231+ishC cells and 231+ishN cells +/- Dox in culture, used to confirm the inducibility of the transfected shRNA constructs. Doxycyclin induces the expression of RFP in the transfected shRNA construct in both Control and Nodal-knockdown cell lines. Nuclei are stained with DAPI (blue) and bars equal 20 μm. (D) Tumor diameter over the course of 4 weeks, following flank injection with 231+ishC or 231+ishN cells. No significant differences in tumor growth were found between 231+ishC or 231+ishN treatments, regardless of Dox administration. Values represent mean tumor diameter (mm) at each time point ± S.E.M. (n≥4, P=0.4). The time at which Dox was administered is indicted by the dotted line.
A. **Immunofluorescence**: Comparison of CD31 expression and Nodal distribution in 231+ishC and 231+ishN cells with and without Dox treatment.

B. **Graph**: Relative CD31 expression (based on average of 5 fields/section) showing a significant decrease in expression with Dox treatment.

C. **Immunofluorescence**: Visualization of CD31 and Nodal expression in 231+ishC and 231+ishN cells with and without Dox treatment.

D. **Graph**: VEGF mRNA expression (fold change relative to 231+ishC-Dox) showing a decrease with Dox treatment.

*Significant difference (p < 0.05)
Figure 3.13 Inducible Nodal inhibition following tumour formation reduces vascularization. (A) Representative immunofluorescence images used to assess CD31 (green) and Nodal (far red) localization. DAPI (blue) was used to stain the nuclei. Merged images are shown for each treatment. Bar equals 50 mm. (B) Relative quantification of CD31 expression in excised 231+ishC and 231+ishN derived tumors. Values represent mean CD31 expression relative to 231+ishC Dox tumors S.E.M., and the asterisk ( ) indicates a significant difference compared to 231+ishC Dox control (n≥4, P=0.04). (C) Representative immunofluorescence images of a 231+shN +Dox tumor in which Nodal was re-expressed. In this case CD31 (green) levels were high in the regions where Nodal (red) was localized. DAPI (blue) was used to stain the nuclei. Bar equals 50 mm. (D) Real time RT-PCR quantification of VEGF expression in 231+ishC versus 231+ishN tumors (+/- Dox). VEGF expression was significantly lower in 231+ishN+Dox tumours compared to all other treatments. Values represent mean VEGF expression relative to 231+ishC Dox tumors S.E.M., and the asterisk ( ) indicates a significant difference compared to 231+ishC Dox control (n≥3, P=0.003).
controls ($n \geq 4$, $p < 0.001$) (Figure 3.14A,B). Additionally, we determined the extent of hypoxia in the tumour sections. For these studies, the mice were injected with Hypoxprobe-1 (pimonidazole hydrochloride) before being sacrificed, and Hypoxprobe-1 adducts, delineating areas of low oxygen, were detected in the tissue sections using immunostaining. Using this method, we determined that hypoxic regions surrounded areas of necrosis, and that 231+ishN +Dox tumours stained more extensively than control tumours (Figure 3.15).

3.5 Discussion

This study demonstrates that expression of the embryonic protein Nodal correlates with microvascular density in human breast cancer tissue, and that Nodal enhances the angiogenic potential of breast cancer cells. Importantly, we discovered that Nodal can be targeted within growing tumours to inhibit vascularization and promote necrosis. Since vascular density is correlated with metastasis and poor prognosis, our study strongly implicates Nodal as a potential prognostic indicator and a novel target for the treatment of breast cancer.

In accordance with previous studies in glioma, Nodal promoted VEGF and PDGF expression in all of the human breast cancer cell lines tested (12). However, we did find that there were differences in protein expression between lysate and conditioned media. For example, T47D cells over-expressing Nodal had elevated VEGF expression in cell lysate, but not in conditioned media. This suggests that Nodal may be involved in regulating secretion of pro-angiogenic factors. Furthermore, although Nodal was consistent in promoting VEGF and
A

- Dox

+ Dox

231+ishC

231+ishN

B

Percent necrosis

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* Significant difference
Figure 3.14 Inducible Nodal inhibition following tumour formation increases necrosis. Doxycyclin-inducible 231+ishC or 213+ishN stably transfected cells were injected with Matrigel into the flanks of nude mice. Mice were given normal chow for the course of the experiment (-Dox), or normal chow for 2 weeks followed by Doxycyclin-chow for 2 weeks (+Dox). After 4 weeks, tumour necrosis was evaluated. (A) Representative H E images of tumours from 231+ishC and 231+ishN cells, with or without Dox chow, demonstrating areas of tumour necrosis. Bar equals 100 mm. (B) Quantification of necrosis corresponding to (A). 231+ishN +Dox tumours exhibited a significant increase in tumour necrosis compared to all other treatment groups. Bars represent mean percent necrosis of total tumour area S.E.M., and the asterisk ( ) indicates a significant difference compared to 231+ishC Dox control (n≥4, P <0.001).
Figure 3.15 Inducible Nodal inhibition following tumour formation increases tumour hypoxia. Doxycyclin-inducible 231+ishC or 213+ishN stably transfected cells were injected with Matrigel into the flanks of nude mice. Mice were given normal chow for the course of the experiment (-Dox), or normal chow for 2 weeks followed by Doxycyclin-chow for 2 weeks (+Dox). Prior to sacrificing animals (at 4 weeks), mice were injected with hypoxyprobe-1 to demarcate hypoxic tissue. Tumour hypoxia was evaluated by staining serial sections with an anti-hypoxyprobe-1 antibody. Images show stained regions of tumour hypoxia (brown) across treatment groups.
PDGF, a full analysis of a pro-angiogenic signature was not performed, leaving the possibility that Nodal may activate other cytokines to mediate vascularization.

When comparing rhNodal rescue treatments from *in vitro* versus *in vivo* experiments, we found that rhNodal differentially rescued the angiogenic phenotype. For instance, addition of rhNodal to shNodal-conditioned media *after* it was collected from the cancer cells *did not* induce a rescue of endothelial cell tube formation *in vitro*. However, addition of rhNodal to shNodal-containing DIVAA angioreactors *did* induce a partial rescue of vascularization. This suggests that rhNodal is able to induce signalling and downstream targets when in contact with cancer cells or with other cell types in the animal, but is not able to propagate signalling when in contact with *only* endothelial cells. We speculate that this is due to differential expression of Nodal receptor components, for example Cripto, across cell types. Although some studies indicate that Nodal may signal in a Cripto-independent manner (30), others suggest that Cripto is required for Nodal-induced phenomena. Moreover, Cripto has been linked to breast and colon tumourigenesis, and one study reported that Cripto promotes HUVEC migration *in vitro* and angiogenesis *in vivo* (31,32). Future studies exploring patterns of Cripto expression on endothelial cells or endothelial progenitors, and the role of Cripto in Nodal-induced angiogenesis would be valuable for developing relevant targeted therapies.

Although we have demonstrated that Nodal induces angiogenesis by regulating classical pro-angiogenic factors and promoting endothelial cell recruitment, it is possible that additional mechanisms of neovascularization also
exist. One possibility is that *in vivo*, bone marrow derived (BMD) progenitor cells are recruited to the tumour. Indeed, BMD endothelial progenitors have been shown to incorporate into the luminal walls of neovessels during breast cancer progression (33). Furthermore, several studies have demonstrated that inhibition of BMD endothelial progenitor cell mobilization/function causes a decrease in tumour vascularization (33,34). Given the role of Nodal in the regulation of stem cell fate and morphogenesis, it is conceivable that it may also regulate tumour vasculogenesis via the recruitment of such endothelial progenitor cells.

In order to recapitulate therapeutic intervention, we designed an assay wherein Nodal was inhibited after tumour initiation. We were able to optimize the timing for activation of our inducible construct so that we minimized confounding factors such as tumour size. We found that there was a decrease in vascularization when Nodal was inhibited in these tumours. In accordance with these observations, one study in melanoma demonstrated a decrease in lung colonization following administration of a function-blocking antibody targeted to Nodal (35). Interestingly, colonies that did form in mice treated with the Nodal antibody exhibited signs of apoptosis inclusive of cytoplasmic swelling and vacuole expansion. However, this study did not include an examination of the anti-angiogenic effects of Nodal inhibition, nor did it explore the possibility that Nodal may be targetable in established lesions that are healthy and proliferative following colonization.

It is not clear from our experiments whether vascular reduction via Nodal inhibition would yield a positive or negative response with respect to metastasis.
Indeed, it has been reported that tumour hypoxia is associated with cellular programmes that yield enhanced metastasis. For instance, it has been shown that tumour hypoxia is associated with elevated tumour initiating cell populations in breast cancer (36), and resistance to chemotherapy and radiation (reviewed in (37) and (38)). Furthermore, over-expression of hypoxia-inducible factor α (HIF-1α) has been shown to promote metastatic phenotypes, and is associated with increased breast cancer relapse and decreased patient survival (39-41). HIF proteins have been shown to up-regulate of a number of stem cell-associated transcription factors that induce tumour-promoting programmes, including TWIST1, TCF3, ZEB1 and ZEB2 (42-46). Paradoxically, although in the current study we show that Nodal inhibition causes decreased VEGF expression and increased tumour necrosis concomitant with tumour hypoxia, we have previously shown that hypoxia induces Nodal expression through HIF-1α (20), and VEGF is one of the best characterized targets of HIF proteins (47). The significance of these contrasting phenomena is unclear at this time. Accordingly, metastatic burden in response to Nodal inhibition, as well as possible combination therapies to circumvent the hypoxic response, should be investigated.

Collectively, we have demonstrated that the stem cell-associated protein Nodal supports a pro-angiogenic niche. This discovery provides mechanistic insight into recent studies demonstrating that cancer cells expressing stem cell-associated genes are better able to recruit vasculature than cancer cells that display a more differentiated phenotype (11). Importantly, we have shown that Nodal is correlated with high vascular density in human breast cancer sections,
and that blocking Nodal expression in established tumours in mice results in a marked reduction in vascularization concomitant with tumour necrosis. Since Nodal is not expressed in non-lactating normal adult breast tissue, our discovery suggests a novel role for Nodal as a tumour-specific target for the treatment of breast cancer angiogenesis.

3.6 References


progression and promotes the growth of prostate cancer cells. *Prostate, 71*: 1198-1209.


CHAPTER 4

Nodal promotes invasive phenotypes via a non-canonical Mitogen

Activated Protein Kinase-dependent pathway
4.1 Abstract

The progression of cancer from localized to invasive disease is requisite for metastasis, and is often characterized by epithelial-to-mesenchymal transition (EMT) and alterations in cellular adhesion and migration. Studies have shown that this transition is associated with an up-regulation of embryonic stem cell-associated genes, resulting in a dedifferentiated phenotype and poor patient prognosis. Nodal is an embryonic factor that plays a critical role in promoting early invasive events during development. Nodal is silenced as stem cells differentiate; however, it plays a specialized role in adult life during placentation and mammary gland development, and an aberrant role during cancer progression. Here, we show that Nodal over-expression in poorly invasive breast cancer and choriocarcinoma cell lines causes increased invasion and migration in vitro. Furthermore, we compare EMT-associated phenotypes in response to Nodal over-expression in both choriocarcinoma and breast cancer cells at the level of gene expression and protein localization. Using Western blot analyses, we show that Nodal promotes these invasive events in both cancer types in part through phosphorylation of ERK1/2. Since Nodal normally signals through SMADs, these findings lend insight into an alternative pathway that is hijacked by this protein in cancer. To evaluate the clinical implications of our results, we show that Nodal inhibition reduces liver tumour burden in a model of spontaneous breast cancer metastasis in vivo, and that Nodal loss-of-function in aggressive breast cancer lines results in a decrease in invasive phenotypes. Our results demonstrate that Nodal is involved in promoting invasion in multiple cellular
contexts, and that Nodal inhibition may be useful as a therapeutic target for patients with progressive disease.

4.2 Introduction

The progression of cancer from localized to invasive disease is requisite for metastasis, and is often characterized by epithelial-to-mesenchymal transition (EMT) and alterations in cellular adhesion and migration. EMT is associated with tissue remodelling during both normal physiological processes such as mammary gland development and placentation, and in cancers, including breast cancer and choriocarcinoma (1,2). Interestingly, while EMT has been thoroughly investigated in breast cancer, its mechanisms are poorly understood in the context of choriocarcinoma.

In many cancer types, cellular invasion and EMT have been linked to the over-expression of embryonic stem cell-associated genes (3,4). Moreover, this stem cell-like gene expression profile is associated with metastasis and poor prognosis. One possible mediator of this invasive cancer signature is Nodal (5,6). Nodal is a member of the transforming growth factor-beta (TGF-β) superfamily, and a morphogen during early embryonic development. Nodal plays an important role in promoting invasive events during primitive streak formation, mammary gland development, and trophoblast invasion during placentation (7,8). It has been postulated that the role that Nodal plays in mediating normal invasive events may similarly manifest during cancer progression.
Indeed, recent studies have shown that Nodal promotes cellular invasion and tumourigenicity in melanoma, prostate cancer, endometrial cancer, and glioma (6,9-14). The role of Nodal in breast cancer and choriocarcinoma is less-characterized in the literature; however, there is evidence that Nodal and its receptors are present in invasive human placental choricarcinoma cell lines and breast cancer cell lines (7,15-17). Furthermore, in breast cancer, Nodal is expressed in invasive and metastatic breast cancer lesions, but it is absent in normal breast tissues (6,7,18). Together these findings provide preliminary evidence that Nodal is correlated with invasive breast cancer and choriocarcinoma cell types; however, the significance of this correlation has not been investigated.

Nodal signals through the activin-like kinase type I (ALK4/7) and type II (ActRIIB) receptor complex, and its signal is enhanced by EGF-CFC family GPI-linked Cripto co-receptor. Activation of this receptor complex causes phosphorylation of SMAD2/3, which then associates with SMAD4. This SMAD2/3/4 complex translocates to the nucleus to regulate transcription of target genes, including NODAL and its inhibitors, LEFTY1/2 (5,19). LEFTY1/2 expression is limited to embryonic contexts and is not expressed in cancer cells, resulting in uncontrolled positive feedback during cancer progression (17).

Non-SMAD pathways activated by Nodal in cancer have been poorly investigated; however, non-SMAD pathway activation in embryology has been reported. For instance, Nodal-induced anterior visceral endoderm (AVE) specification during embryonic patterning is dependent on phosphorylation of p38
Furthermore, phospho-p38 amplifies Nodal signalling during this process, through phosphorylation of the SMAD2 linker region leading to increased SMAD2 activation (20). In cancer, non-SMAD pathway activation by other TGF-β-family proteins is better characterized, revealing possibilities for non-SMAD Nodal targets during disease progression. For instance, the type I receptor has been shown to activate MAPK signalling through ShcA phosphorylation and subsequent interaction with the GRB2/SOS complex in response to TGF-β signalling (21). In fact, both SMAD and ERK signalling are required for TGF-β-induced EMT in keratinocytes (22). Cross-talk between these two pathways has been shown, whereby ERK substrates interact with SMADs to regulate nuclear translocation and gene expression (22). Interestingly, ERK1/2 phosphorylation has also been found to be crucial during trophoblast cell growth and invasion, and ERK activation promotes choriocarcinoma proliferation (23-25). Although Nodal and TGF-β share many signalling commonalities, it is unknown whether Nodal is capable of inducing non-SMAD pathways, like MAPKs, in cancer.

Accordingly, the current study investigates the role of Nodal in breast cancer and choriocarcinoma invasion. First, we demonstrate that in poorly invasive breast cancer and choriocarcinoma cell lines, Nodal promotes cellular invasion and migration, concomitant with an EMT-like phenotype. Conversely, Nodal knock-down results in the opposite phenotype, marked by reduced cellular invasion and a loss of mesenchymal marker expression. Furthermore, we show that these Nodal-induced phenomena are mediated in part through ERK1/2 signalling. In vivo, we demonstrate that inducible Nodal inhibition causes a
reduction in spontaneous metastasis to the liver in NOD/SCID/interleukin-2γ receptor null mice (NSG mice). Our study lends insight into prospective Nodal-targeted therapies for the clinical management of cancer progression.

4.3 Methods

4.3.1 Cell lines and treatments

BeWo human choriocarcinoma cells, and MDA-MB-231, Hs578t, T47D and MCF-7 breast cancer cell lines were obtained from the ATCC and maintained as per instructions. BeWo, T47D and MCF-7 cells were used for gain-of-function models as these cells are poorly invasive and express relatively low levels of Nodal (Figure 2.1A) (26). MDA-MB-231 and Hs578t cells were used for loss-of-function models as these cell lines are highly invasive and express relatively high levels of Nodal (Figure 2.1A). Multiple constructs and reagents were used to knockdown or induce Nodal signalling:

Using expression vectors or recombinant protein for Nodal gain-of-function experiments: T47D and BeWo cells were transfected via Lipofectamine (Invitrogen) with a Nodal expression vector, made with pcDNA 3.3-TOPO cloning kit, or a control pcDNA3.3 vector. H9 hESCs were used to clone the human Nodal open reading frame. Vectors were sequenced and validated, and transfected cells were selected with G418 to generate stable lines. For proof-of-principle, both T47D and MCF-7 cells were also subject to treatment with recombinant human Nodal (rhNodal; 50-100 ng/mL) to activate phospho (P)-SMAD2 signalling as an alternative gain-of-function model (R D
systems). We chose to test this concentration range since previous studies have shown that at least 50 ng/mL of Nodal is required for maintenance of pluripotency in hESCs (27,28). We chose to use 100 ng/mL of rhNodal for our experiments (rather than 50 ng/mL), since we found there was quite a bit of lot-to-lot variance and 100 ng/mL most consistently induced phosphorylation of SMAD2. Furthermore, 100 ng/mL of rhNodal has been used effectively in multiple published studies related to Nodal in cancer (29,30).

**Using shRNA and SB431542 for Nodal loss-of-function experiments:**
MDA-MB-231 cells were transfected via Arrest-In (Open Biosystems) with a pGIPZ lentiviral shRNAmir against Nodal’s 3rd exon (Clone: V2LHS 155453) or a scrambled shRNA Control (Open Biosystems). As an additional loss-of-function model, MDA-MB-231 cells were transfected via Lipofectamine (Invitrogen) with a pTRIPZ lentiviral shRNAmir against Nodal’s 3rd exon (Clone: V2THS 155453) with Tet-On /Tet-Off inducibility or a scrambled inducible shRNA Control (Open Biosystems). All transfected MDA-MB-231 cells were selected with Puromycin to generate stable lines. For both pGIPZ and pTRIPZ knockdown systems, shRNAs targeting at least 4 regions in the Nodal gene were tested for their ability to knockdown Nodal protein expression. For each type of vector, the shRNA achieving the best knock down was chosen. To inhibit Nodal signalling, the ALK4/5/7 inhibitor SB431542 (Sigma) was used at a concentration of up to 10 M. We chose to use 10 M of SB431542 in our experiment, since this dose consistently had the greatest inhibitory effect on SMAD2 phosphorylation, and
has been used in previously published reports on hESCs to block Nodal signalling (27,31).

**Inhibition of the ERK signalling pathway:** To inhibit ERK signalling, U0126 (Sigma), and MEK1/2 inhibitor, was used at a concentration of 10 M. U0126 was given to cells 30 min-1 hr before experiments were performed. To assess the effects of U0126, Western blot for phospho-ERK1/2 (phospho-p44 and phospho-p42) was performed (Cell signalling; Table 4.1).

### 4.3.2 In vitro functional assays

Functional assays were performed on breast cancer or choriocarcinoma cell lines in response to alterations in Nodal and/or ERK1/2 signalling, as indicated in the main text and figure legends:

**2D migration and invasion:** To assess cellular migration, 50,000 cells were seeded onto a Transwell insert, and incubated for 24 hours. Migration was quantified by staining nuclei with DAPI and manual counting of the entire membrane. To assess cellular invasion, the migration protocol was modified by coating Transwells with Matrigel (1:10 Matrigel:Serum-free RPMI) prior to seeding cells. The Matrigel layer was left to solidify overnight at room temperature, and then re-hydrated with serum-free RPMI for one hour before seeding cells.

**3D cell cluster invasion assay:** One million cells (T47D or MDA-MB-231) suspended in 1 mL of complete media (RPMI +10% FBS) were loaded into a bioreactor (Synthecon Rotary Cell Culture System™). The bioreactor was placed
<table>
<thead>
<tr>
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<th>Clone</th>
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Table 4.1 Primary antibodies used for Western blot and immunofluorescence.
in an incubator and set to a rotation of approximately 7 RPM. After 3 days, cell clusters could be seen macroscopically. Clusters were gently removed from the bioreactor using a 10 mL pipette, and placed into a petri dish. Clusters were individually selected, and embedded in bovine collagen type 1 (Invitrogen). Collagen was allowed to solidify at 37 C for 30 min, and was then overlaid with RPMI +10% FBS +treatment (32). MDA-MB-231 clusters were treated with recombinant human Lefty (rhLefty; 1000 ng/mL (17)), an endogenous inhibitor of Nodal in hESCs, and incubated for 36 hours. T47D clusters were treated with 100 ng/mL rhNodal, or 100 ng/mL rhNodal +10 M U0126 MEK1/2 inhibitor, and incubated for 1 week. Following termination of the invasion assays, pictures were taken with an inverted microscope and camera in order to measure distance of invasion from core. 4 measurements evenly spaced around each cluster periphery were averaged to generate a distance score for each cluster. Adobe Photoshop was used to measure distance (Analysis tool).

**Viability:** Viability of endothelial cells in response to conditioned media was assessed using the LIVE/DEAD Viability/Cytotoxycity Kit as per manufacturer's instructions (Invitrogen).

### 4.3.3 Immunofluorescence

Cells were seeded on glass coverslips, fixed with 4% paraformaldehyde, made permeable with 20 mM HEPES and 0.5% TritonX-100, and blocked with serum-free protein block (Dako, Carpinteria, CA) (29). Cells were stained using anti-E-Cadherin antibody (*Table 4.1*), and an Alexafluor secondary antibody
(Invitrogen). DAPI was used to stain nuclei. IgG isotype controls were also included.

### 4.3.4 Real-time PCR

Total RNA was isolated using the Perfect Pure RNA cultured cell kit (5 PRIME) and genomic DNA was degraded using DNase. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using 2 μg cDNA with TaqMan gene expression human primer/probe sets (Table 4.2). All gene expression was normalized to HPRT1.

### 4.3.5 Western blot analysis

Protein lysate was prepared using Mammalian Protein Extraction Reagent (Thermo Scientific), Halt Protease Inhibitor Cocktail (Thermo Scientific), and Phosphatase Inhibitor (Thermo Scientific). Protein was quantified by BCA protein assay (Thermo Scientific) as per manufacturer’s instructions. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, and then transferred onto Immobilon-P membranes (Millipore). Membranes were blocked, and incubated with primary antibody (Table 4.1) and horseradish peroxidase-labelled secondary antibody. Secondary antibodies were detected by Immun-Star Chemiluminescent Detection kit (Bio-Rad) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Images were taken using the ChemiDoc XRS+ System (Bio-Rad) or standard
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Table 4.2 Primer/Probes used for real-time PCR (Applied Biosystems/Invitrogen).
film. For Nodal Western blots, the 39 kDa species was used to assess protein expression as previously described (29). Densitometry was done with ImageJ.

4.3.6 Spontaneous metastasis in vivo

MDA-MB-231 cells were transfected with a doxycyclin (Dox)-inducible Control or Nodal-targeted shRNA. Both constructs expresses RFP upon induction by Dox. 500,000 cells in 100 L RPMI:Matrigel (1:1) were injected into the right thoracic mammary fat pad of 7-8 week old female NSG mice (from D.A.H.). Like NOD/SCID strains, these mice exhibit reduced innate immunity and complete T-cell and B-cell deficiency; however, they also exhibit compromised NK-cell function due to the IL2γR mutation. Dox was administered in mouse chow (0.625 g Dox/1 kg chow) 1 week following injection, after formation of palpable tumour growth. This chow is optimized to deliver 2-3 mg of Dox per day (Harlan Laboratories). Mice were sacrificed when tumours reached 1.3 cm in diameter (termination size varied slightly depending on mouse health or observed depth of tumour growth). Lung and liver were collected from each mouse, fixed with 4% formaldehyde, and stained with H E. All experiments involving animals were approved by the Animal Use Subcommittee at the University of Western Ontario Protocol No. 2008-101 (Appendix 2).

Evaluation of tumour burden in secondary sites: Lung and liver were collected from mice that underwent the spontaneous metastasis assay. The number of mice with macroscopic liver metastases was recorded upon sacrificing animals, since clear differences could be seen in this organ. Tissues were fixed
with paraformaldehyde, paraffin-embedded, sectioned, and stained with H E. For each tissue section, the percent tumour burden was calculated by averaging the tumour area, and dividing it by the average total square area of tissue (Adobe Photoshop). For each animal, an average of 2-3 sections spaced evenly through the tissue was subject to this analysis, to yield one replicate value for percent tumour burden.

4.3.7 Statistical analyses

Parametric data: Statistical analyses of multiple comparisons were performed using a one-way ANOVA followed by a Tukey Kramer Comparisons Post-Hoc test. Parametric data were expressed as mean S.E.M. for replicate values. Non-parametric data: an ANOVA on Ranks followed by the Mann-Whitney rank-sum test was used. Non-parametric data were expressed as median interquartile range. When only two items were compared, a student's t-test was used. All statistical tests were two-sided, and data comparisons were considered statistically significant at $p<0.05$. Statistics were performed using SigmaStat (Dundas Software) in consultation with the biostatistical support unit at the University of Western Ontario.

4.4 Results

4.4.1 Nodal promotes migration and invasion in vitro

Cellular invasion is a complex process that involves active migration and breaching of the extracellular matrix (ECM). We first examined the role of Nodal
signalling in the regulation of cellular migration by performing *in vitro* Transwell chamber assays using breast cancer and choriocarcinoma cell lines. In agreement with previous findings (6,9,29), we confirmed that rhNodal could induce SMAD2 phosphorylation in breast cancer cells (Figure 4.1A). We found that both T47D cells (n=4, p<0.05) and MCF-7 breast cancer cells (n=4, p<0.05) displayed a significant increase in migration when treated with increasing concentrations of rhNodal (0, 50 or 100 ng/mL) (Figure 4.1B,C). In accordance with these findings, when poorly-invasive BeWo choriocarcinoma cells were transfected with a Nodal expression construct (BeWo+Nodal), there was a significant increase in migration through Transwell chambers (n=6, p=0.002) compared to controls (BeWo+Control) (Figure 4.1D,E).

In order to invade and metastasize, a cancer cell must be able to breach the basement membrane of the host organ tissue, and invade through the extracellular matrix (ECM). To test the effects of Nodal on cellular invasion (including ECM degradation), we used a Transwell chamber that was coated with Matrigel, which is a protein mixture used to mimic extracellular matrix and basement membrane. When T47D (n=3, p=0.0038) and MCF-7 (n=3, p=0.004) breast cancer cells were treated with rhNodal (0, 50 or 100 ng/mL), there was a significant increase in cellular invasion at 100 ng/mL rhNodal compared to 0 ng/mL controls in both cases (Figure 4.2A,B). As an alternative method for testing cellular invasion, we employed a 3D invasion assay that more closely resembles an *in vivo* context. Briefly, cells were cultured into 3D clusters in a bioreactor, and then seeded into a collagen plug and treated with rhNodal (0 or
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T47D cells

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B

T47D cells

Cellular Migration (Fold change rel. to 0 ng/mL)

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MCF7 cells

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BeWo cells

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Figure 4.1 Nodal promotes cellular migration in vitro. (A) Western blot validating increased P-SMAD2 in response to treatment with rhNodal in T47D cells. Total SMAD2/3 and β-Actin are used as controls. (B) T47D cells were seeded in Transwell chambers and treated with 0, 50 or 100 ng/mL of rhNodal for 24 hours to assess cellular migration. Cells exhibited a significant dose-dependent upregulation of cellular migration in response to rhNodal (n=4, p<0.05) (C) MCF-7 cells were seeded in Transwell chambers and treated with 0, 50 or 100 ng/mL of rhNodal for 24 hours to assess cellular migration. Cells exhibited a significant dose-dependent upregulation of cellular migration in response to rhNodal (n=4, p<0.05). (D) Western blot validating increased Nodal expression in BeWo cells following transfection with a Nodal expression vector (BeWo+Nodal) versus a control vector (BeWo+Control). The pro-Nodal (39 kDa) band is presented and β-Actin is used as a control. (E) BeWo+Nodal cells or BeWo+Control cells were seeded in Transwell chambers to assess cellular migration after 24 hours. BeWo+Nodal cells exhibited elevated cellular migration compared to BeWo+Control cells (n=6, p=0.002). Data are presented as mean S.E.M for replicate values. Different letters indicate a significant difference.
A. T47D cells

B. MCF7 cells

C. T47D cells (3D)

D. T47D cells

[diagram showing cellular invasion and distance of 3D invasion from core with fold change relative to 0 ng/mL of rhNodal at 0, 50, and 100 ng/mL for both T47D and MCF7 cells.]
Figure 4.2 Nodal promotes cellular invasion in vitro. (A) T47D cells were seeded in Matrigel-coated Transwell chambers and treated with 0, 50 or 100 ng/mL of rhNodal for 24 hours to assess cellular invasion. Cells exhibited a significant upregulation of cellular invasion at 100 ng/mL rhNodal (n=3, p=0.0038) (B) MCF-7 cells were seeded in Matrigel-coated Transwell chambers and treated with 0, 50 or 100 ng/mL of rhNodal for 24 hours to assess cellular invasion. Cells exhibited a significant upregulation of cellular invasion at 100 ng/mL rhNodal (n=3, p=0.004). (C) T47D cells were harvested for 3 days in 3D culture using a bioreactor, and then seeded into collagen type 1 in order to assess 3D invasion over the course of 1 week. Cells treated with 100 ng/mL displayed a significant increase in 3D cellular invasion (measured by the distance cells invaded away from the edge of the cluster) compared to controls (n=10, p=0.032). (D) Representative image showing in vitro 3D collagen invasion assay with T47D cells as described in (C). Bar equals 50 μm and hatched lines delineate the cluster boarder. Data are presented as mean ± S.E.M for replicate values. Different letters indicate a significant difference.
100 ng/mL) for 1 week. We found that T47D cells treated with rhNodal invaded through the collagen, away from the cluster edge, more readily than control cells (n=10, p=0.032) (Figure 4.2C,D).

### 4.4.2 Nodal promotes an EMT-like phenotype

One of the mechanisms underlying cellular invasion in both normal and cancer contexts is EMT. EMT is characterized by a down-regulation of epithelial cell markers, such as E-Cadherin (CDH1), and an acquisition of mesenchymal cell markers, such as Vimentin (VIM), Twist (TWIST1) and N-Cadherin (CDH2) (33). In breast cancers, EMT is also associated with a reduction in Estrogen Receptor (ESR1) expression (34-36). Collectively, this phenotype allows cells to break away from the primary tumour and to invade into secondary sites. Given that Nodal plays a role in promoting morphogenesis in early development, we opted to determine whether Nodal promotes EMT in epithelial-like breast cancer and choriocarcinoma cell lines. Using real-time RT-PCR analysis, we found that rhNodal treatment (100 ng/mL) in T47D and MCF-7 cells caused a down-regulation ESR1 (T47D: n=4, p=0.029; MCF-7: n=5, p=0.008), and an up-regulation of the transcription factor TWIST1 (T47D: n=4, p=0.029; MCF-7: n=3, p=0.036) and the intermediate filament VIM (T47D: n=4, p=0.029; MCF-7: n=5, p=0.008) (Figure 4.3A,B). Similarly, transfection of T47D cells with a Nodal expression construct (T47D+Nodal) resulted in a significant down-regulation of ESR1 (n=3, p=0.030), and an up-regulation of TWIST1 (n=3, p=0.036) and VIM (T47D: n=3, p=0.018) compared to T47D cells transfected with a control vector.
**Figure 4.3 Nodal promotes EMT-like gene expression.** (A) Real time RT-PCR analysis of EMT markers in T47D cells treated with 0, 50, or 100 ng/mL rhNodal (48 hrs). In response to 100 ng/mL of Nodal, T47D cells displayed a decrease in *ESR1* (estrogen receptor) expression (n=4, p=0.029), and an increase in *TWIST1* (n=4, p=0.029) and *VIM* (Vimentin) expression (n=4, p=0.029) compared to controls. *CDH2* (N-Cadherin) and *CDH1* (E-Cadherin) expression did not change (n=4, p 0.05). (B) Real time RT-PCR analysis of EMT markers in MCF-7 cells treated with 0 or 100 ng/mL rhNodal. In response to 100 ng/mL of Nodal, MCF-7 cells displayed a decrease in *ESR1* expression (n=5, p=0.008), and an increase in *TWIST1* (n=5, p=0.036) and *VIM* expression (n=5, p=0.008) compared to controls. *CDH2* and *CDH1* expression did not change (n=5, p 0.05). (C) Western blot validating that Nodal is elevated in T47D cells transfected with a Nodal expression vector (T47D+Nodal) versus a control vector (T47D+Control). The pro-Nodal (39 kDa) band is presented and β-Actin is used as a control. (D) Real time RT-PCR analysis of EMT markers in T47D+Control cells versus T47D+Nodal cells. T47D+Nodal cells displayed a decrease in *ESR1* expression (n=3, p=0.030), and an increase in *TWIST1* (n=3, p=0.036) and *VIM* expression (n=3, p=0.018) compared to T47D+Control cells. *CDH2* and *CDH1* expression did not change (n=3, p 0.05). (E) PCR analysis of EMT markers in BeWo cells transfected with a Nodal expression vector (BeWo+Nodal) versus a control vector (BeWo+Control). BeWo+Nodal cells displayed a decrease in *CDH1* expression (n=4, p<0.001), and an increase in *CDH2* (n=4, p<0.001) and *VIM* expression (n=3, p<0.001) compared to BeWo+Control cells. *TWIST1* expression did not change (n=4, p 0.05). Data are presented as mean S.E.M. for replicate values. Asterisks ( ) indicate a significant different compared to control cells as specified. Expression levels are normalized to *HPRT1*. 
In addition, BeWo+Nodal cells displayed a significant decrease in \( CDH1 \) expression (\( n=4, \ p<0.001 \)), and an increase in both \( CDH2 \) (\( n=4, \ p<0.001 \)) and \( VIM \) (\( n=3, \ p<0.001 \)) expression compared to BeWo+Control cells (Figure 4.3E).

In many instances, EMT is marked by deregulated E-cadherin protein localization, rather than by changes in transcript or protein production. Accordingly, we performed immunofluorescence to determine the localization of E-Cadherin, a pivotal cell-cell adhesion protein in epithelial cells. We found that when T47D or MCF-7 cells were treated with increasing concentrations of rhNodal for 48 hrs, E-Cadherin translocated from the cell membrane to the cytoplasm of the cells (Figure 4.4A,B). Over-expression of Nodal in BeWo cells similarly induced the cytoplasmic localization of E-Cadherin, in addition to down-regulating gene expression (Figure 4.4C).

### 4.4.3 Nodal activates non-SMAD pathways to mediate invasive phenotypes

Given the emerging evidence that implicates Nodal during cancer progression, improving our understanding of both classical and non-classical Nodal signalling mechanisms will be imperative for developing future targeted therapies. As such, we decided to investigate whether Nodal signalling can activate the ERK pathway, as this pathway is highly involved in cellular invasion, proliferation, migration, and differentiation, during both cancer progression and placentation. We first treated T47D cells with rhNodal (100 ng/mL) for 0, 1, 2, 5, 10, or 20 min, and found that Nodal increases P-ERK1/2 in as little as 2 min
Figure 4.4 Nodal promotes cytoplasmic localization of E-Cadherin. (A) Immunofluorescence (IF) showing localization of E-Cadherin (green) in T47D cells or (B) MCF-7 cells after treatment with 0, 50 or 100 ng/mL rhNodal. (C) Immunofluorescence showing localization of E-Cadherin (green) in BeWo cells transfected with a Nodal expression vector (BeWo+Nodal) versus a control vector (BeWo+Control). Nuclei are stained with DAPI (blue) and bars equal 20 μm. All IF was performed 3-4 times.
when replicates were analysed by densitometry (Figure 4.5A,B). Similarly, we treated BeWo cells with rhNodal (100 ng/mL) for 0, 1, 2, 5, 10 or 20 min, and found that Nodal significantly increases P-ERK1/2 by 5 min when replicates were analysed by densitometry (Figure 4.5C,D). In order to determine whether phosphorylation of ERK was dependent on activation of the type I receptor (similar to what has been reported in the literature for TGF-β), we examined the effects of an ALK4/7 inhibitor (SB431542; 10 M, 1 hr) on Nodal-induced ERK1/2 phosphorylation in T47D and BeWo cells. We found that in both cell lines, SB431542 reduced Nodal-induced phosphorylation of ERK1/2 (Figure 4.5E,F). In order to determine if ERK activity may reciprocally regulate SMAD signalling, we treated T47D+Nodal and BeWo+Nodal cells with a MEK inhibitor (U0126; 10 M, 1 hr). We determined that U0126 decreased Nodal-mediated P-SMAD2 in both cases (Figure 4.5G,H). Collectively, our results suggest that through the ALK4/7 receptor, Nodal induces the activation of the ERK1/2 MAPK pathway in breast cancer and choriocarcinoma cell lines. Moreover, Nodal-induced SMAD2 phosphorylation is dependent upon the activation of the ERK1/2 MAPK pathway.

We next wanted to determine the functional significance of Nodal-induced ERK activation. We seeded T47D or BeWo cells into Transwell chambers with Matrigel (for invasion) or without Matrigel (for migration), and treated them with rhNodal alone (100 ng/mL), or with both rhNodal (100 ng/mL) and U0126 (10 M). We found that invasion (T47D: n=3, p=0.017; BeWo: n=3, p=0.022) and migration (T47D: n=3, p=0.010; BeWo: n=3, p=0.005) both increased significantly in response to treatment with rhNodal compared to controls, and this effect was
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P-ERK1/2
ERK1/2
Actin

T47D Parental

B

Densitometry

P-ERK1/2/Total Erk1/2

Exposure to 100 ng/mL rhNodal (min)

-0.5 -0.2 0 0.2 0.5 1 1.5 2 2.5

C

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P-ERK1/2
ERK1/2
Actin

BeWo Parental

D

Densitometry

P-ERK1/2/Total Erk1/2

Exposure to 100 ng/mL rhNodal (min)

0.5 1 1.5 2 2.5 3 3.5 4

E

F

G

H
Figure 4.5 Nodal activates ERK signalling. (A) Western blot time course analysis of ERK1/2 activation in T47D cells following treatment with 100 ng/mL rhNodal for 0, 1, 2, 5, 10, or 20 minutes. P-ERK1/2 increases compared to controls after 2 minutes of rhNodal treatment. Total ERK1/2 and β-Actin are used as controls. (B) Densitometry analysis for all replicate experiments corresponding to (A). ImageJ was used to calculate band density of P-ERK1/2 /band density of total ERK1/2. Data are presented as mean fold change ± S.E.M. for replicate values. Different letters indicate a significant difference compared to controls (n=4, p=0.029). (C) Western blot time course analysis of ERK1/2 activation in BeWo cells following treatment with 100 ng/mL rhNodal for 0, 1, 2, 5, 10, or 20 minutes. P-ERK1/2 increases compared to controls after 5 minutes of rhNodal treatment. Total ERK1/2 and β-Actin are used as controls. (D) Densitometry analysis for all replicate experiments corresponding to (C). ImageJ was used to calculate band density of P-ERK1/2 /band density of total ERK1/2. Data are presented as mean fold change ± S.E.M. for replicate values. Different letters indicate a significant difference compared to controls (n=4, p=0.029). (E) Western blot demonstrating that P-ERK1/2 is elevated in T47D cells transfected with a Nodal expression vector (T47D+Nodal) versus a control vector (T47D+Control), and that phosphorylation is reduced when T47D+Nodal cells are treated with SB431542 (10 M). Total ERK1/2 and β-Actin are used as controls. (F) Western blot demonstrating that P-ERK1/2 is elevated in BeWo cells transfected with a Nodal expression vector (BeWo+Nodal) versus a control vector (BeWo+Control), and that phosphorylation is reduced when BeWo+Nodal cells are treated with SB431542 (10 M). Total ERK1/2 and β-Actin are used as controls. (G) Western blot demonstrating that P-SMAD2 is elevated in T47D+Nodal cells compared to T47D+Control cells, and that this effect is abrogated by treating T47D+Nodal cells with 10 M U0126 (1 hr). Total SMAD2/3 and β-Actin are used as controls. (H) Western blot demonstrating that P-SMAD2 is elevated in BeWo+Nodal cells compared to BeWo+Control cells, and that this effect is abrogated by treating BeWo+Nodal cells with 10 M U0126 (1 hr). Total SMAD2/3 and β-Actin are used as controls.
mitigated by addition of U0126 (Figure 4.6A-D). Importantly, we observed no significant differences in the number of viable cells between treatments for the duration of the invasion and migration assays (24 hrs) (Figure 4.6E-F). In accordance with these results, MCF-7 cells showed similar phenomena in response to rhNodal and U0126 treatments (n=3, p=0.005 for both migration and invasion) (Figure 4.6G,H). Finally, we employed a 3D invasion assay in which a bioreactor was used to generate T47D cell clusters that were subsequently seeded into collagen type 1. Consistent with previous experiments, we found that treatment of clusters with rhNodal caused a significant increase in invasion distance compared to controls, and that this effect was prevented with U0126 (n=14, p<0.001) (Figure 4.7A,B).

In addition to cellular migration and invasion, we wanted to determine whether ERK-activation was involved in Nodal-mediated EMT. First, we added U0126 to MCF-7 cells prior to being treated with rhNodal (100 ng/mL) (extension of experiment represented in Figure 4.3B) and found that U0126 prevented Nodal-mediated induction of TWIST1 and VIM expression as quantified by real time RT-PCR. (Figure 4.8A). Interestingly, ESR1 expression was still upregulated by rhNodal in the presence of U0126 (Figure 4.8A). In accordance with these results, in choriocarcinoma cells, addition of U0126 to BeWo+Nodal cells (extension of experiment represented in Figure 4.3E) resulted in a decrease of Nodal-induced CDH2 and VIM expression, and an increase of CDH1 expression back to control levels (n=4, p 0.05) (Figure 4.8B). To complement these studies, we also performed immunofluorescence to determine the
Figure 4.6 Nodal-induced cellular invasion and migration is mediated by ERK. (A) T47D cellular invasion through a Matrigel-coated Transwell chamber increased in response to 100 ng/mL rhNodal (n=3, p=0.017), and this effect was mitigated back to control levels by the addition of 10 μM U0126. (B) BeWo cellular invasion through a Matrigel-coated Transwell chamber increased in response to 100 ng/mL rhNodal (n=3, p=0.022), and this effect was mitigated back to control levels by the addition of 10 μM U0126. (C) T47D cellular migration through a Transwell chamber (without Matrigel) increased in response to 100 ng/mL rhNodal (n=3, p=0.010), and this effect was mitigated back to control levels by the addition of 10 μM U0126. (D) BeWo cellular migration through a Transwell chamber (without Matrigel) increased in response to 100 ng/mL rhNodal (n=3, p=0.005), and this effect was reduced by the addition of 10 μM U0126 (n=3, p=0.008, b vs. c). (E) T47D viability assay and (F) BeWo viability assay demonstrating equal cell viability after 24 hours between treatments corresponding to (A-D). (G) MCF-7 cellular invasion through a Matrigel-coated Transwell chamber increased in response to 100 ng/mL rhNodal (n=3, p=0.005), and this effect was mitigated by the addition of 10 μM U0126. (H) MCF-7 cellular migration through a Transwell chamber (without Matrigel) increased in response to 100 ng/mL rhNodal (n=3, p=0.005), and this effect was mitigated by the addition of 10 μM U0126. Data are presented as mean ± S.E.M. for replicate values. Different letters indicate a significant difference as specified.
A

Distance of 2D invasion from core (mm)

T47D  T47D  T47D
+ rhNodal  + rhNodal + rhNodal + U0126

B

T47D cells

Control  + 100 ng/mL rhNodal  + 100 ng/mL rhNodal + 10 μM U0126
Figure 4.7 Nodal-induced cellular invasion through collagen is mediated by ERK. T47D cells were harvested for 3 days in 3D culture using a bioreactor, and then seeded into collagen type 1 in order to assess 3D invasion over the course of 1 week. Distance of invasion was recorded as an average of 4 measurements evenly spaced around the cluster periphery. (A) Cells treated with 100 ng/mL rhNodal displayed a significant increase in 3D cellular invasion compared to controls (n=14, p<0.001), and this effect was reduced back to control levels in response to treatment with 10 μM U0126. Data are presented as mean ± S.E.M for replicate values, and different letters indicate a significant difference. (B) Representative images from 3D invasion assay corresponding to data presented in (A). Bar equals 50 μm and hatched lines delineate the cluster borderer.
Figure A: mRNA Fold Change (Relative to T47D +0ng/mL rhNodal)
- ESR1
- CDH1
- TWIST1
- CDH2
- VIM

Figure B: mRNA Fold Change (Relative to BeWo+Control)
- CDH1
- TWIST1
- CDH2
- VIM

* Denotes significant difference (p < 0.05)
Figure 4.8 Nodal-induced changes in EMT marker expression are mediated by ERK. (A) Real time RT-PCR analysis of EMT markers in MCF-7 cells exposed to 100 ng/mL rhNodal alone, or 100 ng/mL rhNodal + 10 μM U0126 (48 hrs). Treatment with rhNodal alone caused decreased ESR1 expression (n=5, p=0.008), and increased TWIST1 (n=3, p=0.036) and VIM expression (n=5, p=0.008). Addition of U0126 rescued TWIST1 and VIM expression back to control levels. ESR1 expression was not rescued with the addition of U0126 (n=5, p=0.008). CDH2 and CDH1 expression did not change with any of the treatments. (B) Real time RT-PCR analysis of EMT markers in BeWo cells transfected with a Nodal expression vector (BeWo+Nodal) versus a control vector (BeWo+Control), or in BeWo+Nodal cells treated with 10 μM U0126 (48 hrs). BeWo cells expressing Nodal displayed a decrease in CDH1 expression (n=4, p<0.001), and an increase in CDH2 (n=4, p<0.001) and VIM expression (n=3, p<0.001) compared to BeWo+Control cells. Treatment of BeWo+Nodal cells with U0126 rescued CDH2 and VIM expression back to BeWo+Control levels, and significantly reverted CDH1 expression to near-control levels (n=4, p=0.029). TWIST1 expression did not change with any of the treatments. Data are presented as mean ± S.E.M. for replicate values. Asterisks (*) indicate a significant different compared to control cells as specified. Expression levels are normalized to HPRT1.
localization of E-Cadherin in response to U0126. We found that pre-treatment of T47D cells and MCF-7 cells with U0126 (10 M) followed by the addition of 100 ng/mL rhNodal (1 hour) resulted in a full (T47D) or partial (MCF-7) rescue of E-Cadherin from the cytoplasm to the cell membrane (Figure 4.9A,B).

4.4.4 Nodal inhibition impairs invasion in vitro in part through decreased P-ERK

In a complementary set of loss-of-function experiments, we wanted to determine if Nodal inhibition could decrease the invasiveness of more basal-like breast cancer cells, which express high levels of endogenous Nodal. Using a Transwell chamber assay with Matrigel (for invasion) or without Matrigel (for migration), we found that knocking down Nodal expression in MDA-MB-231 cells with shRNA (231+shNodal) resulted in a significant decrease in both migration (n=5, p<0.001) and invasion (n=5, p=0.016) compared to 231+shControl cells (Figure 4.10A-C). When MDA-MB-231 cells or Hs578t cells were treated with SB431542 ALK4/7 inhibitor (0, 1, or 10 M), a similar reduction in cellular invasion was observed (n=5, p<0.05 for both cell types) (Figure 4.10D-F). Lastly, when we performed a 3D invasion assay through collagen using MDA-MB-231 cells treated with rhLefty (1000 ng/mL), we found that the distance of invasion was significantly reduced compared to controls (n=7, p=0.001) (Figure 4.10G,H).

We also investigated whether Nodal inhibition in MDA-MB-231 cells could alter phosphorylation of ERK to mediate the observed changes in cellular invasion and migration. We confirmed via Western blot that 231+shNodal cells
100 ng/mL rhNodal + 10 μM U0126

MCF-7

E-Cadherin DAPI Merge

0 ng/mL rhNodal

100 ng/mL rhNodal

100 ng/mL rhNodal + 10 μM U0126

T47D

E-Cadherin DAPI Merge

0 ng/mL rhNodal

100 ng/mL rhNodal

100 ng/mL rhNodal + 10 μM U0126

MCF-7

E-Cadherin DAPI Merge

0 ng/mL rhNodal

100 ng/mL rhNodal

100 ng/mL rhNodal + 10 μM U0126
Figure 4.9 Nodal-induced cytoplasmic localization of E-Cadherin is mediated by ERK. (A) Immunofluorescence (IF) showing localization of E-Cadherin (green) in T47D cells and (B) MCF-7 cells after treatment with 0 ng/mL rhNodal, 100 ng/mL rhNodal, or 100 ng/mL rhNodal + 10 μM U0126. Nuclei are stained with DAPI (blue) and bars equal 20 μm. All IF was performed 3-4 times.
A

B

MDA-MB-231 cells

C

MDA-MB-231 cells

D

E

MDA-MB-231 cells

F

Hs578t cells

G

MDA-MB-231 cells (3D)

H

MDA-MB-231

Distance of 3D invasion from core

Control rhLefty (1000 ng/mL)

Control + 1000 ng/mL rhLefty
Figure 4.10 Nodal inhibition impairs invasion and migration in vitro. (A) Western blot validating decreased Nodal expression in MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) versus a scramble control shRNA (231+shControl). The pro-Nodal (39 kDa) band is presented and β-Actin is used as a control. (B) Cellular migration and (C) invasion through Matrigel of 231+shControl versus 231+shNodal cells was quantified following a 24 hr incubation. Cellular migration and invasion were significantly reduced in response to Nodal knockdown compared to controls (n=5, p<0.02). (D) Western blot validating that treatment of MDA-MB-231 cells with SB431542 (0, 1, or 10 M) causes a dose-dependent decrease in SMAD2 phosphorylation. SMAD2/3 and β-Actin are used as loading controls. (E,F) Cellular invasion assay (24 hours) through a Matrigel-coated Transwell chamber of (E) MDA-MB-231 cells or (F) Hs578t cells treated with 0, 1 or 10 M of SB431542. Cellular invasion was significantly reduced in both cell lines in response to SB431542 (n=5, p<0.05). (G) 3D invasion assay demonstrating that rhLefty (1000 ng/mL) inhibits invasion in MDA-MB-231 cells. Bars represent relative invasion relative to untreated controls. (H) Representative images corresponding to (G). Bar equals 50 μm and hatched lines delineate the cluster border. All data are presented as mean ± S.E.M for replicate values, and different letters indicate a significant difference.
displayed reduced P-ERK1/2 compared to 231+Control cells, and that addition of rhNodal (100 ng/mL) to 231+shNodal cells rescued P-ERK1/2 (Figure 4.11A). In accordance with these results, we found that rhNodal (100 ng/mL) could restore cellular invasion and migration in 231+shNodal cells to levels observed in 231+shControl cells, but that this ability of rhNodal to rescue invasion and migration was mitigated when downstream ERK1/2 activation was prevented with U0126 (10 M) (Figure 4.11B-D). Furthermore, we found that inhibition of ERK1/2 phosphorylation in 231+shControl cells with U0126 resulted in a significant decrease in cellular invasion (n=3, p<0.001) and migration (n=3, p=0.002), and that these effects of U0126 were not rescued by the addition of rhNodal (Figure 4.11B-D). It should be noted that the number of viable cells did not change across treatments (Figure 4.11E). Hence our results indicate that Nodal regulates breast cancer invasion via a downstream ERK1/2-dependent pathway.

Since Nodal over-expression caused an EMT-like event in poorly aggressive cell types, we tested whether Nodal inhibition could affect the expression of mesenchymal phenotypes in MDA-MB-231 cells. Using PCR analysis we found that treatment of MDA-MB-231 cells with SB431542 (10 M) resulted in a decrease in TWIST1 (n=3, p<0.001), CDH2 (n=3, p<0.001), and VIM (n=3, p=0.001) expression, but did not affect epithelial marker expression (including CDH1 or ESR1) (Figure 4.12A). Similarly, 231+shNodal cells displayed a significant reduction in TWIST1 (n=3, p<0.001), CDH2 (n=3, p<0.001), and VIM (n=3, p=0.031) expression, but did not display altered CDH1
Figure 4.11 Nodal inhibition impairs invasion *in vitro* in part through decreased P-ERK1/2. (A) Western blot analysis of MDA-MB-231 cells transfected with a Control (231+shControl) or a Nodal-targeted shRNA (231+shNodal) showing that phosphorylation of ERK1/2 decreases when Nodal is reduced. Addition of recombinant human Nodal (rhNodal; 100 ng/mL) restores ERK1/2 phosphorylation in 231+shNodal cells. Total ERK1/2 and β-Actin are used as controls. (B) Western blot analysis validating that U0126 reduces phosphorylation of ERK1/2 in parental MDA-MB-231 cells. Total ERK1/2 and β-Actin are used as controls. (C) Cellular invasion (through Matrigel) and (D) cellular migration (no Matrigel) of 231+shControl or 231+shNodal cells through a Transwell chamber in the presence or absence of rhNodal (100 ng/ml) U0126 (10 M) (24 hrs). Cellular invasion and migration were significantly reduced in 231+shControl cells treated with U0126, and this was not rescued with rhNodal. Cellular invasion and migration were significantly decreased in 231+shNodal cells as compared to 231+shControl cells, and the inclusion of rhNodal rescued invasion and migration in 231+shNodal cells to control levels. This ability of rhNodal to rescue the effects of shNodal on invasion and migration was mitigated by U0126 (n=3, different letters are significantly different, p<0.001). (E) Bar graph summarizing the results of LIVE/DEAD Cytotoxicity/Viability assays (Invitrogen). Viability was constant across all MDA-MB-231 treatment conditions after 24 hours (corresponding to C and D) (n=8).
A

[B431542] on MDA-MB-231 cells

B

C

kDa

231+shControl 231+shNodal

140-

Vimentin

57-

Slug

30-

N-Cadherin

42-

Actin
Figure 4.12 Nodal inhibition reduces expression of mesenchymal markers in MDA-MB-231 cells. (A) Real time RT-PCR analysis of EMT markers in MDA-MB-231 cells treated with SB431542 small molecule inhibitor (0 or 10 M, 48 hrs). In response to SB431542, which inhibits ALK4/7, MDA-MB-231 cells displayed reduced expression of TWIST1 (n=3, p<0.001), CDH2 (n=3, p<0.001), and VIM (n=3, p=0.001). CDH1 and ESR1 did not change in response to SB431542. All data are presented as mean ± S.E.M for replicate values, and asterisks (*) indicate a significant difference compared to 0 M controls as specified. (B) Real time RT-PCR analysis of EMT markers in MDA-MB-231 cells stably transfected with a Nodal-targeted shRNA (231+shNodal) or a scrambled Control shRNA (231+shControl). 231+Nodal cells displayed reduced expression of TWIST1 (n=3, p<0.001), CDH2 (n=3, p<0.001), and VIM (n=3, p=0.031). CDH1 and ESR1 were not significantly different between 231+shNodal cells and 231+shControl cells. All data are presented as mean ± S.E.M for replicate values, and asterisks (*) indicate a significant difference compared to 231+shControls as specified. (C) Western blot showing that 231+shNodal cells exhibit lower protein expression for mesenchymal markers N-Cadherin, Vimentin, and Slug, compared to 231+shControls. β-Actin is used as a control.
or ESR1 expression compared to 231+shControl cells (Figure 4.12B). In accordance with these results, Western blot analysis revealed that mesenchymal markers N-Cadherin, Vimentin and Slug were lower in 231+shNodal cells compared to 231+shControl cells (Figure 4.12C). Taken together, while we did find that Nodal inhibition reduced mesenchymal marker expression in highly aggressive MDA-MB-231 cells, we did not find any alterations in epithelial marker expression, indicating that Nodal knockdown is not enough to induce a full mesenchymal-to-epithelial transition in poorly differentiated cell lines.

4.4.5 Nodal inhibition reduces spontaneous metastasis of breast cancer to the liver

In corroboration with our in vitro assays, we performed a spontaneous metastasis assay using highly invasive MDA-MB-231 breast cancer cells that express high basal levels of Nodal. MDA-MB-231 cells were transfected with a Nodal-targeted doxycyclin (Dox)-inducible shRNA construct (231+ishN), or a control Dox-inducible shRNA construct (231+ishC) (Figure 4.13A,B), and injected into the mammary fat pads of 7-8 week old NSG mice. Dox was administered in mouse chow one week after injections. Tumours were palpable (2 mm diameter) at this time. Once tumours reached 1.3 cm in diameter (end point varied depending on depth of tumour), which took approximately 5 weeks, mice were sacrificed, and lungs and livers were collected.

Upon examination of lung tissue stained with H&E, we saw no difference in the percentage of tumour burden in the lung across treatments (Figure
A

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Nodal
Actin

B

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-Dox
+Dox
Figure 4.13 Validation of inducible shRNA transfection into MDA-MB-231 cell lines. (A) Western blot analysis demonstrating that MDA-MB-231 cells transfected with a Nodal-targeted doxycyclin (Dox)-inducible shRNA (231+ishN) downregulate Nodal protein expression upon administration of Dox compared to MDA-MB-231 cells transfected with a scrambled control Dox-inducible shRNA (231+ishC). (B) Confocal images of 231+ishC cells and 231+ishN cells +/- Dox in culture, used to confirm the inducibility of the transfected shRNA constructs. Doxycyclin induces the expression of RFP in the transfected shRNA construct in both control and Nodal-knockdown cell lines. Nuclei are stained with DAPI (blue) and bars equal 20 μm.
Interestingly, tumour burden ranged from approximately 10%-95%, indicating that in many cases, lung tissue was not over-saturated with tumour. Although macroscopically, we found no difference in lung tissue upon sacrificing the mice, we did see robust differences in liver metastases, whereby 231+ishN +Dox groups had macroscopically observable lesions in only 1/10 mice (compared to 7/11 for 231+ishC Dox, 6/10 for 231+ishC +Dox, and 11/15 for 231+ishN Dox) (Figure 4.15A). Accordingly, we stained liver tissue with H E and measured the percentage of tumour burden per liver section across treatments. We found that there was a significant decrease in extent of liver metastases in 231+ishN +Dox mice compared to all other treatment groups (Figure 4.15B).

### 4.5 Discussion

Here we show for the first time that Nodal promotes invasive phenotypes in both breast cancer and choriocarcinoma cell lines. We reveal a link between Nodal and the induction of EMT-like phenomena in multiple cell types to yield a more aggressive phenotype both in vitro and in vivo. These observations are in accordance with others that have found a link between Nodal and cancer invasion, for example, during glioma invasion or melanoma progression (10,12), and with previous work from our laboratory demonstrating a link between Nodal and breast cancer progression in xenograft models (37). Indeed, previous studies have found that Nodal is rare in radial growth phase melanoma, but is frequently expressed during vertical growth phase melanomas and in melanoma...
A

- Dox

+ Dox

231+ishC

231+ishN

(B) Percent tumour burden

(Average of 3 sections per lung lobe per mouse)
Figure 4.14 Nodal inhibition does not affect spontaneous metastasis of breast cancer to the lung. 500,000 231+ishC or 231+ishN cells were injected into the right thoracic mammary fat pad of NSG mice. Doxycyclin was administered 1 week following injection. Mice were sacrificed when tumors reached 1.0-1.5 cm in diameter and lung and livers were analysed macroscopically or using H&E stained sections. (A) Images of lung sections stained with H&E across treatment groups. Bar equals 1 mm. (B) Quantification of tumor burden in lungs show no significant differences between treatment groups (n=10). Each point represents the average tumor burden for one animal and the filled diamond indicates the median value. Different letters indicate a significant difference.
A

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![Images of liver sections](image)

B

| Percent tumour burden (Average of 3 sections per liver sample per mouse) |
|-----------------------------|-----------------------------|
|                            | 231+ishC                     |
|                            | 231+ishC -Dox                |
|                            | 231+ishC +Dox                |
|                            | 231+ishN                     |
|                            | 231+ishN -Dox                |
|                            | 231+ishN +Dox                |

![Graph showing tumour burden](image)

Legend:
- a
- b
Figure 4.15 Nodal inhibition reduces spontaneous metastasis of breast cancer to the liver. 500,000 231+ishC or 231+ishN cells were injected into the right thoracic mammary fat pad of NSG mice. Doxycyclin was administered 1 week following injection. Mice were sacrificed when tumors reached 1.0-1.5 cm in diameter and lung and livers were analysed macroscopically or using H&E stained sections. (A) Images of livers from mice injected with 231+ishC or 231+ishN cells and fed either normal or Dox-containing chow. Metastases have been outlined by a white dashed line and bar equals 1 mm. (B) Quantification of tumor burden in livers shows a significant decrease in metastasis in 231+ishN +Dox mice compared to all other groups (n=10, p<0.05). Each point represents the average tumor burden for one animal and the filled diamond indicates the median value. Different letters indicate a significant difference.
metastases (12). Furthermore, Nodal is highly expressed in invasive breast cancer lesions (7). The current study mechanistically addresses these previous correlations by demonstrating that Nodal promotes and sustains invasive phenotypes.

We found that Nodal supports cellular invasion through altered expression of epithelial and mesenchymal cell markers in both choriocarcinoma and breast cancer cell lines. This adds Nodal to a growing list of TGF-β family members that have been shown to regulate EMT in normal epithelial cells and during cancer progression. For example, TGF-β promotes EMT in a variety of cell types including breast cancer cells, pulmonary epithelial cells and keratinocytes (38), and differentiation factor-9 (GDF-9) has been shown to induce EMT in prostate cancer (39). Interestingly, GDF is a member of the TGF-β family that has been shown to dimerize with Nodal in the embryonic node to mediate Left-Right asymmetry (40). As it has been shown that EMT is associated with invasive phenotypes, and that EMT is often induced by stem cell-associated proteins, it fits that our findings implicate Nodal during this complex transition.

We determined that Nodal promotes cellular invasion and EMT-like phenomena via the activation of the ERK1/2 MAPK pathway in breast cancer and choriocarcinoma. Prior to this study, the effects of Nodal on cellular behavior during development and in cancer were largely attributable to the induction of SMAD signalling. However, many TGF-β superfamily proteins, which share similarities between type I and type II receptor components, employ non-SMAD pathways during signal propagation. Specifically, TGF-β has a profound effect on
activation of ERK signalling, through phosphorylation of ShcA and activation of the GRB2/SOS complex via its type I receptor (21). Moreover, TGF-β promotes EMT at least in part via ERK1/2 MAPK signalling (38). For example, TGF-β was shown to induce ERK and p38 signalling in human skin keratinocyte cells (HaCaT), ras-transfected H357 keratinocytes, and malignant Il-3 keratinocytes to induce EMT (22); and inhibition of MEK with U0126 reversed TGF-β-induced EMT in H357 and Il-3 cell lines (22). Cripto, Nodal’s co-receptor protein, can also signal through ERK (41). In one study, NCCIT teratocarcinoma cells were shown to have high basal levels of P-ERK1/2 as well as high Cripto expression. Cripto knockdown in these cells significantly impaired P-ERK1/2, and this was rescued with the inclusion of soluble Cripto (41). Moreover, treatment of MCF-10A cells (which exhibit undetectable Cripto) with rhNodal did not stimulate proliferation (indicative of ERK signalling); however, over-expression of Cripto in MCF-10As rendered cells susceptible to rhNodal-induced proliferation (41). Together, these studies suggest that multiple mechanisms of MAPK activation, perhaps receptor-mediated either through ALK or Cripto proteins, may explain our findings that Nodal can activate non-SMAD pathways, specifically ERK, to elicit its effects.

Our results suggest there may be multiple points of crosstalk between the SMAD2/3 and ERK1/2 pathways. First, we demonstrate that addition of rhNodal to cells causes an upregulation of phosphorylated ERK1/2. Based on reports of TGF-β-induced ERK activation, this may be an indirect effect via receptor phosphorylation of an upstream mediator of the ERK cascade (such as ShcA) (21), or a direct effect via receptor phosphorylation of ERK1/2 (42). Second, we
show that MEK inhibition leads to a decrease in Nodal-induced phosphorylation of SMAD2. As mentioned, previous studies in development have shown that the SMAD2/3 linker region is receptive to phosphorylation by MAPK pathways, specifically phospho-p38 (20). ERK1/2 MAPKs may similarly phosphorylate SMAD2/3 to facilitate Nodal-induced signalling. Third, we show that MEK inhibition reduces transcription of Nodal-induced $TWIST1$. In agreement with previous findings, this suggests that ERK may alter SMAD-mediated gene expression, either through regulating translocation of SMAD proteins to the nucleus, or by directly interfering with gene transcription (43). We also found that MEK inhibition rescued Nodal-induced mislocalization of E-Cadherin. In accordance with these observations, previous reports have shown that inhibition of the ERK pathway is required for relocalization of E-Cadherin to the plasma membrane after internalization during EMT (44,45).

An important finding in our study is that Nodal inhibition in highly invasive breast cancer cells can revert cellular behavior to a less-aggressive state, and impair breast cancer metastasis to secondary organs. These results mirror the effects observed in our gain-of-function models, suggesting that Nodal-induced phenotypes may be reversible in advanced disease. In accordance with these observations, one group found that tumour supressor zinc-α2-glycoprotein (ZAG) was able to reduce cellular invasion through induction of TGF-β-mediated MET in pancreatic ductal adenocarcinoma, marked by a downregulation of $VIM$, and upregulation of $KRT19$ (Keratin19) and $CDH1$ expression (46). Interestingly, ZAG-induced MET was mediated via modulation of TGF-β-mediated ERK2
phosphorylation (46). These findings closely mirror our findings with Nodal inhibition in MDA-MB-231 cells, whereby invasion is correlated with Nodal expression, and Nodal inhibition causes a reduction in mesenchymal markers concomitant with a decrease in Nodal-induced P-ERK1/2. These findings provide multiple possible targets and time points for clinical intervention in patients with Nodal-positive tumours.

In our NSG mouse model, we observed a marked difference in Nodal-mediated tumour burden between lung and liver breast cancer metastases. Specifically, we saw that alterations in Nodal expression in MDA-MB-231 cells had no effect on lung metastasis, while it did for liver metastasis. There are a number of possibilities for this outcome. First, Nodal may regulate factors that differentially mediate organ-specific metastasis. For example, it has been shown that Claudin-2 is required for the initiation and growth of breast cancer liver metastases; however, interestingly, cell lines generated from aggressive breast cancer liver metastases with elevated Claudin-2 expression show decreased spontaneous metastasis to the lung by more than 3-fold (47). Other studies have reported differences in expression of chemotactic signalling axes between cancer cells and specific organ destinations. For example, CXCR4 is known to be elevated in breast cancer, while its ligand, CXCL12, is elevated in common sites of breast cancer metastasis, including liver, bone marrow and lung (48,49). It is possible that Nodal supports tumour metastasis to specific sites through regulating factors associated with organ-specific metastasis.
Another possible explanation for the differences in lung versus liver metastasis is that the NSG mouse model is highly permissive to metastasis due to a severely reduced immune system. In the human condition, this hypothesis is supported by the fact that organ transplant recipients undergoing immune-suppression therapies have an elevated incidence of cancer (50,51). Moreover, differences in tumour-initiation have been reported between different model organisms with different immune system backgrounds. For example, Morrison and colleagues have shown that melanoma cells have elevated tumour growth and tumour-initiating frequency in NSG mice (approximately 25% of cells injected) compared to NOD/SCID mice (approximately 0.0001% of cells injected) (52,53). In accordance with these findings, it has been reported that tumour cells generated in immunocompromised mice do not form tumours in syngeneic immuno-competent mice; whereas, tumour cells generated in immuno-competent mice can form tumours in both immuno-competent or immunocompromised mice (54). Whether there is a connection between Nodal expression and immune surveillance during cancer progression is currently unknown.

Taken together, our results demonstrate that Nodal promotes invasive phenotypes in both breast cancer and choriocarcinoma cell lines. We found that Nodal supports cellular invasion through altered expression of epithelial and/or mesenchymal cell markers, and that Nodal can activate non-SMAD pathways, specifically ERK, to elicit its effects. Most importantly, we show that Nodal inhibition in highly invasive cells can revert cellular behaviour to a less-aggressive state, and reduces breast cancer metastasis to the liver. Our findings
lend insight to recent studies that implicate Nodal in the progression of various types of cancer, and provide multiple possible targets to which clinical intervention might be effective.

4.6 References


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CHAPTER 5

General Discussion and Conclusions
5.0 General Discussion and Conclusions

This study reveals, for the first time, that the embryonic protein Nodal mediates metastatic phenotypes in breast cancer. It is well-documented that the acquisition of embryonic signatures is associated with cancer progression. Although previous studies have shown that Nodal contributes to a malignant phenotype (1-5), the studies in this thesis link Nodal to a unique variety of cellular functions that have not been previously explored, including novel mechanisms of non-SMAD signalling. I have also demonstrated that cellular functions classically attributed to Nodal in embryonic contexts, such as cellular invasion, are maintained in breast cancer (6). I have also demonstrated that Nodal mediates multiple phenotypes that are mechanistically divergent, albeit interconnected, in accordance with the principle that embryonic proteins can have widespread and adverse effects when they usurp adult tissue homeostasis. Taken together, my research has elucidated novel contributions of the embryonic protein Nodal to the metastatic dissemination of breast cancer, and has exposed many interesting new questions about Nodal-mediated events in breast cancer.

5.1 Novel roles for Nodal in cancer

This study demonstrates multiple novel roles for Nodal during cancer progression. First, I have shown that Nodal has a robust effect on breast cancer angiogenesis, and that the expression of Nodal correlates positively with vascular density in human breast cancer lesions. A previous report by Hueng et al., titled “Inhibition of Nodal suppresses angiogenesis and growth of human gliomas”,
demonstrated that Nodal is correlated with VEGF in human gliomas, and that Nodal knockdown leads to reduced tumour size and vessel length (measured in tissue sections) in vivo (7). However, this report did not demonstrate the effects of Nodal on angiogenesis, as the title and contents of the paper suggest (7). Angiogenesis is a process, and although a tumour’s vascular density and VEGF expression are indicative of angiogenic potential, they are not a measure of functionality. Moreover, measurements of vessel length in random fields of view do not necessarily account for vessel density. Rather, according to the International Consensus on Evaluation of Angiogenesis in Solid Human Tumors, grading of angiogenic “hotspots” is a more accurate method to assess tumour vascularization. By this consensus, vessel grading is based on the number of vessels in one hotspot, and is evaluated irrespective of vessel length. Although the hotspot method is admittedly simplified (8), there is little evidence to support the notion that longer vessels are worse for cancer outcome than smaller vessels, or that vessel length alone is a reliable prognostic indicator. In fact, studies have reported the opposite correlation, that vessel length is significantly lower in tumours compared to corresponding normal tissue (9). Indeed, all studies that explore the relevance of vessel length quantify per unit volume of tissue, since 2D sections are not appropriate for assessing a 3D phenomenon (such as length) (8,9). In this study, the hotspot method was used to determine that Nodal is correlated with high tumour vascular density, thereby providing more reliable and clinically-relevant evidence that Nodal is associated with the angiogenic progress.

In light of these arguments, I have shown here for the first time, a functional connection between Nodal and angiogenesis. In vitro, Nodal promotes tube formation
and migration of multiple endothelial cell types, including human adult microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs). In vivo, Nodal promotes vascular recruitment in multiple animal models, and inducible Nodal inhibition following tumour formation significantly reduces vascular density. We found that Nodal elicited its angiogenic effects through production of pro-angiogenic proteins; however, the effects we saw in vivo were quite robust, and therefore may be mediated by additional unexplored mechanisms. One possibility is that Nodal promotes mobilization of bone marrow derived cells (BMDCs) to contribute to de novo vessel formation. Indeed, it has been reported that endothelial progenitor cells from bone marrow incorporate into breast tumour-associated neovessels (10). Furthermore, many studies have demonstrated that impaired BMDC recruitment disrupts tumour vascularization in vivo (10,11). Given that Nodal mediates stem cell fate and plays a directive role during embryonic morphogenesis, it is conceivable that it may also regulate progenitor mobilization from the bone marrow to support tumour vascularization.

Another novel finding in this study is that Nodal potentiates ERK signalling in breast cancer and choriocarcinoma cells. I have demonstrated that Nodal induces phosphorylation of ERK1/2 in poorly invasive breast cancer and choriocarcinoma cell lines, and that phosphorylation is mitigated when the Nodal type I receptor is inhibited. Conversely, when ERK signalling is inhibited with a MEK inhibitor, Nodal-induced phosphorylation of SMAD2 is reduced. This demonstrates a link between canonical Nodal signalling and ERK in cancer. In accordance with these results, studies have reported signalling crosstalk between SMAD2/3 and ERK pathways. Studies with TGF-β
have shown that the type I receptor can phosphorylate both ShcA and ERK1/2 (12,13). Furthermore, the linker region of SMAD2/3 has been shown to be receptive to phosphorylation by p38 MAPK signalling (14): It is possible that ERK can phosphorylate SMAD2/3 by a similar mechanism.

Functionally, I have demonstrated that Nodal-induced ERK signalling in part mediates invasive phenotypes and EMT in breast cancer and choriocarcinoma cell lines that are otherwise poorly invasive and well-differentiated. In accordance with these findings, in normal mammary gland epithelial cells TGF-β-induced EMT has been shown to be dependent on ERK signalling inclusive of a reduction of cell surface E-Cadherin (15). However, my studies are the first to report a link between Nodal, ERK and EMT in both breast cancer and in choriocarcinoma. Future studies should examine if this fundamental discovery also applies to other phenomena where Nodal is present and when cellular migration and EMT occurs, such as embryonic morphogenesis, mammary gland development, and endometrial cycling (3,16).

5.2 Classical Nodal-associated phenotypes are recapitulated in breast cancer

In addition to the novel roles for Nodal revealed in this study, phenotypes that have been previously associated with Nodal in other cancer types were confirmed for the first time in breast cancer. For example, I show here that Nodal promotes elevated proliferation:apoptosis ratios in breast cancer cell lines, both in vitro and in vivo. In accordance with these findings, Hardy et al. showed that Nodal was correlated with aggressive phenotypes in melanoma, and that Nodal expression was induced by Notch4. Interestingly, this study demonstrated that the Notch4/Nodal relationship alters
proliferation:apoptosis ratios in favour of growth, and supports the acquisition of aggressive phenotypes in poorly aggressive melanoma cells (17). This study did not investigate whether Nodal could alter proliferation:apoptosis ratios irrespective of Notch4 status (17). Interestingly, it has been shown that Notch4 is not expressed in T47D breast cancer cells (18), yet I have shown that Nodal does indeed mediate changes in proliferation:apoptosis ratios in this cell line. This suggests that Nodal may regulate the proliferative capacity of these cells in a Notch-independent manner.

In contradiction with these results, several studies by Peng and colleagues have reported that Nodal promotes apoptosis and inhibits proliferation in trophoblast, breast cancer, and ovarian cancer cells (19-21). The key experimental difference in these studies is that Nodal was *overexpressed* in cells that express high endogenous Nodal, whereas here, Nodal was *inhibited* in cells that express high endogenous Nodal. Furthermore, Peng and colleagues use 500 ng/mL of recombinant mouse Nodal on cells that express *high Nodal*, which is 5-fold higher than the dose of recombinant human Nodal (rhNodal) used in this thesis on cells that express *low Nodal* (21). 500 ng/mL is also more than 5-fold higher than the dose of Nodal required to maintain pluripotency in hESC cultures (22,23), indicating that this concentration may be beyond physiological relevance. Alternatively, assuming that cell viability was incorporated as a control (as it should always be), these data raise the curious possibility that a concentration-dependent biphasic mechanism might be at play, similar to the function of TGF-β (24,25). To address this possibility, I performed Transwell migration assays on T47D and MCF-7 breast cancer cells treated with 0, 50, 100, or 500 ng/mL of rhNodal, and I found that migration was mitigated at 500 ng/mL (**Figure 5.1**). In agreement with
A

Cellular Migration (Fold change rel. to 0 ng/mL)

T47D migration

<table>
<thead>
<tr>
<th>[rhNodal] (ng/mL)</th>
<th>0</th>
<th>50</th>
<th>100</th>
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B

Cellular Migration (Fold change rel. to 0 ng/mL)

MCF-7 migration

<table>
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<tr>
<th>[rhNodal] (ng/mL)</th>
<th>0</th>
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<td>Cellular Migration</td>
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Figure 5.1 Nodal has a biphasic concentration-dependent effect on cellular migration. (A) Experiment corresponding to Figure 4.1B, with an additional treatment (500 ng/mL rhNodal) incorporated into the data set. T47D migration through a Transwell chamber was significantly lower in response to 500 ng/mL rhNodal, compared to 100 ng/mL (p=0.015, n=4). (B) Experiment corresponding to Figure 4.1C, with an additional treatment (500 ng/mL rhNodal) incorporated into the data set. MCF-7 migration through a Transwell chamber was significantly lower in response to 500 ng/mL rhNodal, compared to 100 ng/mL (p=0.005, n=4). Different letters indicate a significant difference of p<0.05 (unless indicated otherwise). Bars represent mean ± S.E.M. for replicate values.
these findings, another member of our lab performed the same experiment on HTR-8/SVneo trophoblast cells, and found similar results (16). This suggests that although high Nodal expression is correlated with tumour growth, very high doses of Nodal may have the opposite effect. A wider range of Nodal concentrations should be used in future experiments to improve our understanding of this impending phenomenon. However, again, it should be noted that it is unclear whether these high doses of Nodal are physiologically relevant.

Invasion is another phenotype associated with Nodal in multiple contexts, that has likewise been linked to Nodal in breast cancer and choriocarcinoma in this study. I have shown that Nodal promotes cellular invasion and migration in poorly aggressive breast cancer and choriocarcinoma cell lines. Conversely, I have shown that Nodal inhibition reverts invasive phenotypes in highly aggressive breast cancer cell lines. In accordance with these observations, Nodal has been linked to cellular invasion in melanoma and glioma (1,2), and previous studies from our laboratory have shown that Nodal is correlated with invasion in trophoblast cell lines (16). Interestingly, Peng and colleagues have reported seemingly contradictory results, whereby Nodal was shown to inhibit trophoblast cell migration and invasion (26). However, as mentioned in the previous discussion about proliferation:apoptosis, this may be explained by the possibility that Nodal may induce a biphasic effect on cell invasion and migration (16). However, future experiments ought to be performed to validate these speculations.
5.3 Cancer phenotypes are connected

Phenotypes associated with cancer progression work in concert, and connections between the phenotypes explored here have been reported at length in the literature. Bone marrow-derived cells, in particular, seem to be a major driving force that connects multiple aspects of tumourigenesis, as they contribute to the composition of the tumour stroma and the pre-metastatic niche (27-30). Given that Nodal has a widespread effect on breast cancer phenotypes, it is valuable to consider what “master regulators” of disease progression might be receptive to Nodal signalling. Note that the following discussion is based largely on breast cancer, since breast cancer constitutes the majority of this thesis, and a large portion of the choriocarcinoma study was contributed by Guihua Zhang.

5.3.1 Links between tumour growth and angiogenesis

5.3.1.1 Tumour mass dormancy and the angiogenic switch

That tumour growth is dependent on the ability to recruit a vasculature has been reported extensively in the literature with respect to multiple different tumour types, at both primary and secondary sites (11,31-34). The inability of tumours to growth beyond a certain size due to insufficient vascularization is called Angiogenic Dormancy, or Tumour Mass Dormancy (35). At secondary sites, tumour mass dormancy is characterized by metabolically active avascular micrometastatic lesions that do not grow beyond 1-2 mm in diameter (in accordance with the diffusion limit of oxygen (36)) due to a balance between proliferation and apoptosis. The angiogenic switch marks the transition out of this dormant state, at which point metastases are said to be “macro”
(i.e. larger than 1-2 mm due to vascular infiltration), and exhibit elevated proliferation rates compared to apoptosis (31,35,36). In this study (Chapter 2), I have demonstrated that Nodal is required for the transition from micro to macrometastatic growth, marked by a switch to a proliferation:apoptosis ratio (quantified by Ki67:TUNEL) greater than 1. Furthermore, I have shown that Nodal promotes a robust angiogenic phenotype in breast cancer (Chapter 3). It is possible that the mechanism of growth transition at secondary sites is due to a transition in angiogenic switching, mediated by Nodal expression. Indeed, when von Willebrand factor (vWF) was used to qualify vascularization in micrometastases (data not shown), I noticed that metastases formed by cells in which Nodal was knocked down frequently exhibited lower vascular infiltration. These data are in accordance with those reported by several research programmes, including those of Judah Folkman, Shahin Rafii, Vivek Mittal, and David Lyden (note: these names have been listed without permission from the individuals).

5.3.1.2 Bone marrow-derived cells mediate the angiogenic switch

One mediator of tumour mass dormancy is recruitment of bone marrow derived cells. Based on the finding that Nodal promotes angiogenesis, it is plausible that Nodal mediates recruitment of bone marrow-derived progenitor cell types to promote an angiogenic phenotype. In an elegant experiment by Gao et al. published in Science in 2008, MMTV-PyMT mice were reconstituted with GFP+ bone marrow, and pulmonary metastases were analysed. Results showed that micrometastases were avascular, while macrometastases exhibited high vascularity with GFP+ EPC incorporation (11). Interestingly, inhibition of one protein, Id1, prevented the transition from micro to
macrometastatic growth, by blocking EPC recruitment and the angiogenic switch (11). These results mirror my finding that Nodal inhibition prevents the switch from micro to macrometastatic growth in the lung (Chapter 2). It is possible that like Id1, Nodal mediates the angiogenic switch at secondary sites through recruitment of EPCs. Indeed, based on my results, BMDC mobilization is now being investigated in our laboratory.

5.3.2 Links between angiogenesis and invasion

5.3.2.1 Bone marrow-derived myeloid cells promote MET

In this study I have shown that Nodal promotes EMT in poorly aggressive breast cancer and choriocarcinoma cell lines, and that Nodal inhibition promotes a loss of mesenchymal markers (a partial MET) in highly aggressive breast cancer cell lines. Furthermore, Nodal was associated with increased liver metastases in a mouse model of spontaneous metastasis. Although it is well-documented that EMT mediates invasion and metastasis to secondary sites, studies have (paradoxically) shown that secondary metastases often display an epithelial-like phenotype (37-39). This suggests that while EMT events early in the metastatic cascade are requisite for dissemination and translocation of tumour cells, MET events later in the metastatic cascade are important for secondary tumour growth. In other words, phenotypic switching by tumour cells, rather than EMT specifically, is a necessary component of successful metastasis. Gao et al. recently published an article in Cancer Research that examined this phenomenon, in light of the fact that bone marrow derived cells play a critical role in the angiogenic switch to macrometastases. They found that during breast cancer metastasis, bone
marrow derived myeloid progenitor cells were recruited to the pre-metastatic lung. Upon the arrival of breast cancer cells, these myeloid progenitor cells were able to induce mesenchymal-to-epithelial transition of tumour cells through *downregulation* of SMAD2 signalling, to promote a switch to macrometastatic growth (37). Given that Nodal is a marker of pluripotency in embryos, and that Gao showed that downregulation of SMAD2 signalling mediated MET in secondary lesions, it is possible that Nodal regulates these switching events in cancer cells. Consistent with this hypothesis and with Gao’s observations, studies have reported that subpopulations of breast cancer cell lines undergo phenotypic switching, marked by shifts in CD44 and CD24 expression, in a Nodal/Activin-dependent manner (40).

### 5.3.2.2 Tumour-associated macrophages promote vascularization and secrete proteolytic enzymes

Macrophages are another tumour-associated cell type (also known as *TAMs*) that have a profound effect on both cellular invasion and angiogenesis (41-44). In a study published in *Cancer Cell* by Hanahan and colleagues, gene expression analyses were performed at progressive stages of pancreatic cancer in RIP1-Tag2 mice (which spontaneously develop pancreatic islet tumours due to expression of the SV40 T antigen) (42). They found that macrophage-derived cysteine cathepsins were upregulated during disease progression, and played a major role during the angiogenic switch and tumour cell invasion (42). In another study by Condeelis’ research group, gene expression analyses were performed on breast TAMs, which revealed that TAM-expression profiles resembled developmental signatures (45). Condeelis’ group also
demonstrated that triple-clustering of TAMs, endothelial cells, and a pro-invasive protein called MENA is predictive of metastatic disease, a prognostic indicator Condeelis calls **TMEM** (29). In accordance with these findings, I have shown here that Nodal regulates both angiogenesis and tumour cell invasion in breast cancer. Furthermore, I have shown that Nodal expression is correlated with vascular density in human tissue sections demarcated by CD31. It is possible that Nodal mediates these phenotypes through regulation of tumour-TAM interactions, especially since TAM recruitment is often associated with an embryonic signature (45).

### 5.3.2.3 Proteolytic enzymes

One connective feature of both cellular invasion and angiogenesis that was not explored in this study, but that would be worth investigating in the future, is proteolytic degradation. I have demonstrated that although Nodal promotes both migration and invasion through Transwell chambers, higher doses of rhNodal are required to affect invasion. This finding was recapitulated in both T47D and MCF-7 poorly aggressive breast cancer line, treated with increasing doses of rhNodal (Chapter 4). The key difference between migration and invasion assays is that invasion requires proteolytic degradation through the Matrigel layer on the Transwell membrane. Proteolysis is a key step in cellular invasion to mediate degradation of the basement membrane and the ECM. Proteolytic degradation of the ECM is also important during angiogenesis to make room for sprouting vasculature. It is plausible that Nodal regulates proteolytic networks (at higher concentrations than are required for migration) to dually mediate its invasive and angiogenic effects. In support of this hypothesis, studies have shown that TGF-β1
promotes MMP expression in highly aggressive MDA-MB-231 breast cancer cells and inhibits expression of MMP-inhibitor, RECK, in an ERK-dependent manner (46). Given the similarities between Nodal and TGF-β, and that Nodal regulates ERK signalling in breast cancer cells, a similar mechanism might be used by Nodal to regulate invasion and/or angiogenesis; however, this has yet to be investigated.

5.3.2.4 Tumour hypoxia

Hypoxia is a phenomenon that occurs once tumours grow beyond the limits of oxygen diffusion (36), that upregulates both pro-invasive and pro-angiogenic programmes. Indeed, cellular invasion and angiogenesis are two of the most commonly studied hypoxia-induced phenotypes. In a previous report from our laboratory published in *Molecular Biology of the Cell*, we showed that hypoxia induces Nodal expression to upregulate invasion, migration, and tube formation in otherwise poorly-aggressive breast cancer cell lines (47). Furthermore, Nodal knockdown by shRNA prevented the induction of these phenotypes under hypoxic conditions. It is possible that in my *in vivo* studies, tumour hypoxia increased the expression of Nodal thereby promoting metastasis and angiogenesis. Furthermore, given the ability of Nodal knockdown to prevent angiogenesis and metastasis (hypoxia-induced phenomena), it is possible that Nodal is a pivotal regulator of certain hypoxia-induced phenotypes.
5.3.3 Links between tumour growth and invasion

5.3.3.1 ERK regulates migration and proliferation

MAPK signalling has long been associated with promoting proliferation and invasion in breast cancer (48). Here I have shown that Nodal induces a non-canonical ERK MAPK signalling pathway to promote cellular invasion. This is not surprising given the role of TGF-β in promoting invasion and proliferation through SMAD-ERK crosstalk in keratinocytes and epithelial cells (12,49). I have also shown that Nodal-induction of ERK causes invasion through EMT and upregulation of TWIST1 and VIM. In accordance with these findings, a previous study reported that TGF-β-induction of MAPKs, including ERK, causes phosphorylation and stabilization of TWIST1, resulting in EMT of breast cancer cells (50). Of note, Massagué and colleagues have published extensively on the topic of non-canonical TGF-β signalling in cancer, and have found that MAPKs are major mediators of TGF-β’s tumourigenic effects (51). In cancer, mutations in the ERK pathway have been associated with progression of colon cancer, leukemias, and skin cancers, among others (52-54). Although I have shown that Nodal promotes invasive phenotypes in part through ERK signalling, whether Nodal also mediates its proliferative effects through alterations in ERK signalling remains elusive.

5.3.3.2 Integrins and adhesion proteins

In an experiment that was not included in this study, I performed an adhesion assay that revealed a significant decrease in ECM-adhesion in 231+shNodal cells compared to 231+shControls (Figure 5.2). In particular, adhesion to fibronectin changed significantly, suggesting, perhaps, a shift in integrin expression in Nodal-
Relative Adhesion (Colourmetric assay)

- BSA (ve)
- Fibronectin
- Collagen I
- Collagen IV
- Laminin I
- Fibrinogen

231+shControl
231+shNodal
Figure 5.2 Nodal inhibition alters cellular adhesion to ECM proteins. MDA-MB-231 cells were transfected with a control shRNA (231+shControl) or a Nodal-targeted shRNA (231+shNodal) and subjected to a CytoSelect™ ECM Cell Adhesion Assay (CellBiolabs). There was a significant decrease in adhesion to Fibronectin (p=0.001), Collagen I (p=0.001) and Collagen IV (p=0.001) in 231+shNodal cells compared to 231+shControl cells. No significant changes in adhesion to Laminin I (p=0.062) or Fibrinogen (p=0.105) were seen between treatments (n=4). Data are presented as mean ± S.E.M for replicate values.
expressing versus Nodal-knockdown cell lines. Integrins are a family of cell surface adhesion proteins that are associated with cellular migration, proliferation, and apoptosis (55). Several studies have demonstrated that breast cancer progression is associated with increased expression of β3 integrin (56-58). One such study reported that the pro-tumourigenic effects of TGF-β during breast cancer progression, including EMT and metastasis, was mediated in part by interactions between the TGF-β Type II receptor and β3 integrin (56). Another study demonstrated that αvβ1 was correlated with lymph node metastases in breast and pancreatic cancer patients (59). Given that integrins play a regulatory role in both proliferation and invasion of cancer cells, these reports suggest that Nodal and integrin regulation may be worth future investigation.

5.3.3.3 Cripto is associated with invasion and proliferation in cancer

One important aspect of Nodal signalling is Cripto, and it is likely that the effects of Nodal signalling in breast cancer depend on the presence of this co-receptor on tumour cells and/or tumour-associated cell types. During embryonic axis patterning, Cripto mediates Nodal signalling, differentiating its promiscuous SMAD2/3 pathway from other TGF-β and Activin ligands (60,61). Studies have shown that Cripto is overexpressed in a subset of primary melanomas and cell lines, and that Cripto expression is correlated with invasive and proliferative phenotypes (62). Cripto expression is also associated with tumourigenesis in breast and oral cancers (63-66). Cripto mediates its mitogenic and invasive effects through activation of Nodal-independent Ras/MAPK and PI3K/Akt signalling pathways, in addition to stimulating Nodal-dependent SMAD2/3 (63). Given that Nodal signals in cancer depend on Cripto
expression, consideration of Cripto functionality may be useful for understanding how to effectively target Nodal during cancer progression.

5.4 Conclusions

Nodal is an embryonic protein that links multiple aspects of tumourigenesis at both primary and secondary sites. In this study I have demonstrated both novel and classical roles for Nodal during breast cancer: I have revealed a novel link between Nodal and angiogenesis, ERK signalling and EMT. I have also shown that classical Nodal-induced phenotypes, such as cellular invasion, exist in breast cancer and choriocarcinoma as they do in other types of cancer. My research has raised many curious questions that are worth further investigation, including the role of Nodal in regulating BMDC recruitment, proteolytic degradation, integrin expression, and tumour-stroma interactions. Given that Nodal is normally restricted to embryonic or highly specialized adult contexts, targeting Nodal expression in breast cancer poses an exciting avenue for therapeutic intervention.

5.5 References


APPENDICES
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This approval is valid from **November 1, 2009 to October 31, 2010**

The protocol number for this project remains as **2008-101**

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   - If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
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**c.c. Approved Protocol**

- L. Postovit, W. Lagerwerf

**Approval Letter**

- L. Postovit, W. Lagerwerf
CURRICULUM VITAE

Daniela Quail, Ph.D. Candidate
Schulich School of Medicine and Dentistry, Dept. of Anatomy and Cell Biology
The University of Western Ontario, London, ON, Canada, N6A 5C1
Tel: (226) 448-6490  Email: dquail@uwo.ca

EDUCATION BACKGROUND

Ph.D. Candidate, The University of Western Ontario, Jan 2008-June 2012 .
Faculty of Medical Science, Department of Anatomy and Cell Biology
Dr. Lynne-Marie Postovit (Supervisor)
Dr. D. Laird, Dr. P.K. Lala, Dr. A. Allan (Advisors)
The role of Nodal in breast cancer metastasis and plasticity.

Honours Specialization in Biology, Minor in Philosophy

Faculty of Science, Department of Biology
Dr. J.P. Wiebe (Supervisor)
Dr. K. Hill and Dr. M. Bernards (Advisors)
Insulin-like growth factor-1 (IGF-1) affects progesterone metabolizing enzyme expression and activity in MCF-7 breast cancer cells.

AWARDS AND SCHOLARSHIPS

- CBCF Doctoral Fellowship, 2010-2012 (accepted)
- CIHR Doctoral Scholarship, 2010-2012 (awarded and declined)
- CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) scholarship recipient, 2009-2011
- Poster presentation award, CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) and the Department of Oncology, 7th Annual Oncology Research and Education Day, 2010
- 1st place platform presentation, CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) and the Department of Oncology, 6th Annual Oncology Research and Education Day, June 12, 2009.
- 1st place platform presentation, Lawson Health Research Institute, Victoria Research Laboratories Research Day, March 24, 2009
- Ontario Graduate Scholarship (OGS) recipient, 2009-2010 (Declined)
- Natural Sciences and Engineering Research Council of Canada- Canada Graduate Scholarship (NSERC- CGS) recipient, 2009-2010
- Graduate Thesis Research Award 2008-2010

*indicates first author, or presenter
• Ontario Graduate Scholarship (OGS) recipient, 2008-2009
• Translational Breast Cancer Studentship from the London Regional Cancer Program (TBCRU) recipient, 2008-2009
• Western Graduate Research Scholarship, 2008-2012
• Schulich Graduate Scholarship, 2008-2012
• Dean's Honour List with distinction, The University of Western Ontario, 2006-2007
• Graduated with Honours from the University of Western Ontario, 2003-2007
• Dean's Honour List with distinction, The University of Western Ontario, 2005-2006
• First class honours entry scholarship, The University of Western Ontario, 2003

REFEREEED PUBLICATIONS

Accepted manuscripts:

Manuscripts in revisions, or recently submitted:
Quail DF*, Zhang G, Hess DA, and Postovit LM. Nodal promotes invasive phenotypes via a non-canonical Mitogen Activated Protein Kinase-dependent pathway. [In revisions, Oncogene]

Ongoing studies:
Quail DF, Zhang G, Kwan K, Brackstone M, and Postovit LM. Nodal expression in DCIS with microinvasion in human breast cancer. [In progress]
Quail DF, Zhang G, Hess DA, and Postovit LM. Nodal promotes stem cell phenotypes in breast cancer. [In progress]

*indicates first author, or presenter
PATENTS

Targeting Nodal as a modulator of angiogenesis and/or neovascularization in established tumours. Review of Invention (ROI). [Submitted to World Discoveries UWO Tech ID: W-12-020] Inventors: Lynne-Marie Postovit, Daniela Quail, David Hess

CONFERENCE ABSTRACTS AND PRESENTATIONS


Quail, D* and Postovit, L.M. (2009) Nodal promotes metastasis and plasticity in breast cancer cells [Platform presentation]. CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) and the Department of Oncology, 6th Annual Oncology Research and Education Day, London, ON, Canada (Internal conference)


*indicates first author, or presenter
NON-REFEREED CONTRIBUTIONS


MEMBERSHIPS

American Association of Cancer Research (AACR), 2009-2013

RELEVANT EMPLOYMENT EXPERIENCE

Teaching Assistant, 2009-2012
Medical Science 4930 (4th year undergraduate course)
- Responsible for designing marking schemes, marking assignments, and following up with students regarding assignment material

Dr. J.P. Wiebe, Department of Biology, The University of Western Ontario
- Designed and conducted experiments related to progesterone metabolism in breast cancer

RELEVENT EXTRACIRRICULAR EXPERIENCE

Member of the Graduate Student Council, 2008-2009
- Anatomy and Cell Biology student representative
- Act as a liaison between students from the Department of Anatomy and Cell Biology, and The University of Western Ontario Society of Graduate Studies (SOGS) student council.

Executive member of the Cancer Awareness Society (UWOCAS), 2006-2008.
- Responsible for coordinating all events and fundraisers, and collaborating with companies and organizations in the London community.
- Organized various public events with the Canadian Cancer Society, such as Climb for the Cure, Locks of Love, and not-for-profit concerts for cancer featuring local bands from the London community.

Involved in cancer-related events at Western and in the broader London community.
- Relay for Life participant and organizer at UWO and Richmond Hill, 2004-2008
- Canadian Cancer Society volunteer, 2006-2008
- CIBC Run for the Cure participant, 2005-2008

Mentor for first-year undergraduate female science students at Western through Women in Science and the Department of Mathematics (WISDOM), 2006-2007.

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