Development and Cancer:
The role of Twisted Gastrulation in mammary gland development and in cancer.

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Cynthia Louise Forsman-Earl

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Anna Petryk, Advisor

November, 2014

Acknowledgements

In the following passages I try to thank all those who assisted in this endeavor and who I will always owe a debt of gratitude. There are many more not mentioned here who were also important to my success. They offered support and sometimes just a "hang in there" at the right moment or a thoughtful question at seminar that moved a project forward, to this supportive community, I thank you.

This thesis truly is the result of the significant and valuable collaboration of many persons who have assisted me in its achievement. In the case of colleagues involved with the research for chapter 2, they have already been recognized as authors on that publication. There are many more who deserve acknowledgement and I will attempt to acknowledge them here.

The Petryk lab served as an active source of insight and challenge that has refined my thinking and without whom this thesis simply would not exist. I thank my advisor Anna Petryk, without her willingness and support to pursue new projects, this work and my career as a cancer researcher simply would not have been possible. Furthermore, her guidance and provision of forward momentum is largely responsible for my complicated but eventual success. Truly, her hard work and dedication have ensured my continued existence in the scientific life. I also wish to express my deepest and sincerest gratitude to Kaylee Schwertfeger who generously opened her lab and resources to ensure my success. She has seen me through tears of joy and agony and to her, my heartfelt thanks. I want to also thank Schwertfeger lab members; Nick Brady, Laura Bohrer, Maria Farooqui, Polly Chuntova and Johanna Reed for spirited and helpful discussion. In the Petryk lab, I thank, Brian Schmidt and Ashley Petersen, whose efforts to provide an efficient lab was instrumental in the accomplishment of this work. I would also like to thank my fellow students BreAnne MacKenzie and especially Charlie Billington Jr. The accomplishment of this magnitude, by necessity, is a journey that is highlighted by incredible highs and crushing lows. To have a trench mate that so inspired, motivated and supported me was invaluable. I would also like to express appreciation for the efforts of Brandon Ng, Nermin Shenouda, Nevin Shenouda, Julie Herder, Lauren Jelenchick, Jim Omlie, Claire Kuo, Michael Capp, Rachel Heinze, Grace Goepfrich and Tyler Burgoyne.

The Developmental Biology Center labs were a constant source of inspiration and support. I particularly want to thank Mu Sun, formerly of Michael O' Connor's lab, for allowing me to collaborate on one of his projects. I also want to thank Dr. Hiroshi Nakato who took a chance and hired me as a lab technician. That position eventually led me to pursue my PhD in the MCDB&G program. I thank all other Developmental Biology center labs, including particularly the members of the labs of Mike O' Connor, Jeff Simon, Laura Gammill, Hiroshi Nakato, Yasu Kawakami and Tom Neufeld for their support over the years.

I owe much to the labs of Kim Mansky and Raj Gopalakrishnan where I have benefitted from the help of Eric Jensen, Ann Emery, Lan Pham and Aaron Broege.

I want to thank the members of my committee for their insights and interest in my work as well as for my development as a scientist. Michael O' Connor (chair), Yasuhiko Kawakami, David Zarkower and Kim Mansky were extremely helpful. Their willingness to review drafts, attend presentations, challenge my thinking and encourage me forward is largely responsible for the success I have enjoyed thus far. I appreciate the commitment of Douglas Yee to serve on my committee and as a co-mentor on my Department of Defense Predoctoral Breast Cancer Research Fellowship that funded much of my graduate career. Again, I thank Anna Petryk and Kaylee Schwertfeger for all the opportunities you have made possible.

On a personal note, I thank my children; Tasha, Amanda and Danielle Forsman-Earl. You have grown into the most loving, supportive adults a mother could ever hope for. Thank you for your understanding and support as I know the late arrivals and cancellations were many. I thank my mother, Marjorie Forsman, who kept my home and life afloat when the waters threatened to close over my head. I thank Kimberly Ahmann, my partner in all things, who stepped into the breach at a moment's notice and always had just the right words to put life back into perspective. Truly, you are some of my most pivotal collaborators and were as critical to my success as my scientific ones.

Dedication

This thesis is dedicated to my family for this achievement would not have been possible without their love and support. It does not escape my attention that you remained positive and supporting through the long days and nights of my graduate education. Thank you for your love and support as I worked to achieve this accomplishment that was so important to me.

Abstract

It is becoming increasingly clear that the molecular mechanisms that maintain and promote pluripotency during development reemerge as important mediators of tumorigenicity. Bone morphogenetic proteins (BMPs) and their modulators play numerous and diverse roles during development and, as recent evidence suggests, in cancer. One key modulator of BMP signaling during craniofacial and mammary gland development is the glycoprotein Twisted gastrulation-1 (TWSG1). The loss of *Twsg1* results in craniofacial malformations, a delay in mammary gland development and lactation defects.

TWSG1 has been shown to function both in the positive and negative regulation of BMP signaling. This dual nature may be explained first by TWSG1's ability to bind BMPs and prevent their interaction with receptors. This function is similar to other extracellular BMP antagonists such as Noggin and Gremlin. Additionally, the TWSG1: BMP ligand complex can be joined by Chordin and when this tripartite complex is formed Tolloid can cleave Chordin releasing BMPs into the extracellular space at some concentration. The timing of this release and availability of other extracellular partners may, in part, dictate the influence TWSG1 has on BMP signaling. Interestingly, *TWSG1* overexpression has been detected in tumors of tissues in which it also plays a key role in development such as the oral cavity and breast. It therefore provides an interesting model by which to investigate the tenuous balance between pluripotency and tumorigenesis.

Table of Contents

List of Figures

List of Tables

Chapter 1: Background and Significance

Mammary Gland Development

It is becoming increasingly clear that the inappropriate reactivation of developmental signaling pathways play a significant role in the development and progression of breast and other cancers (Lu et al., 2013; Riggs et al., 2012; Vogelstein and Kinzler, 2004). Understanding normal mammary gland development and the required molecular interaction provides insight into the detection and treatment of breast cancer. In both human and mouse, the mammary gland is a dynamic organ that responds and develops over the lifetime of the female. Embryonic development establishes the rudimentary ductal tree that during puberty, pregnancy and lactation undergoes complex morphological changes. Precise molecular control and timing of these signaling pathways is required at all stages.

In the mouse, mammary gland development begins around embryonic day 10.5 with the formation of the presumptive mammary line along each flank of the developing embryo, running between fore and hind limb buds (Watson and Khaled, 2008). Over the next 24 hours, placodes, consisting of a thickened and slightly raised lens shaped patch of morphologically distinct ectodermal cells, arise in a spatially and temporally restricted manner. The specification and spatial location of placode formation is dependent upon interactions between the epithelial and mesenchymal compartments.

As development progresses, the cells of the placode arrange themselves

concentrically and at embryonic day 12 can be seen as small elevations along the milk line known as mammary buds (Watson and Khaled, 2008). The bud begins to invaginate into the underlying mesenchyme and at which time the mesenchymal cells condense, elongate and orient in a radial fashion around the invading epithelium. At the end of invagination, the epithelium resembles a hanging ball with a stalk of epidermal like cells maintaining a connection to the future nipple closely surrounded by mammary mesenchymal cells (Figure 1). Interactions between epithelial and mesenchymal cells continue to be critical at this stage of development. The bud remains rather static for the next 48 hours and then at embryonic day 16.5 the bud forms a primary sprout, which is the first step in forming the rudimentary ductal tree. At about the same time, the fat pad begins to condense. As the embryonic developmental period draws to a close the mammary bud has invaginated into the mesenchyme, further elongated to sprout into the stromal fat pad and has begun branching to form a rudimentary ductal tree with approximately 13-20 branches. As in humans, the mammary gland remains relatively quiescent until the onset of ovarian hormone production during puberty.

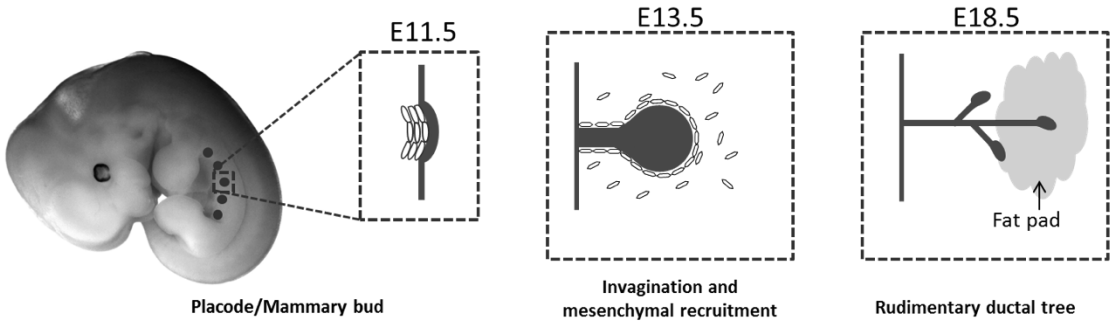

Figure 1: Mammary gland development in the mouse during embryogenesis. Mammary gland development at E11.5, E13.5 and E18.5 highlighting the formation and elongation of the epithelial duct.

At the onset of puberty, around 5-6 weeks of age, the ductal tree within the mammary gland responds to sex hormones by forming terminal end buds that elongate and branch, filling the fat pad. These buds consist of bipotent cap cells, which are thought to generate both myoepithelial and luminal epithelial cells. The luminal epithelial cells generate the mass of the duct and some subset of these cells undergo apoptosis to form the lumen. Further development of the ductal tree occurs in response to pregnancy hormones (Figure 2). After parturition and the cessation of lactation, involution of the ductal network occurs returning the gland to a near virgin morphology. The dynamic changes seen in mammary gland are regulated by a diverse set of morphogens, growth factors, and hormones. Integration and regulation of these signals is required for the proper establishment of mammary gland anlagen and subsequent development through puberty and pregnancy.

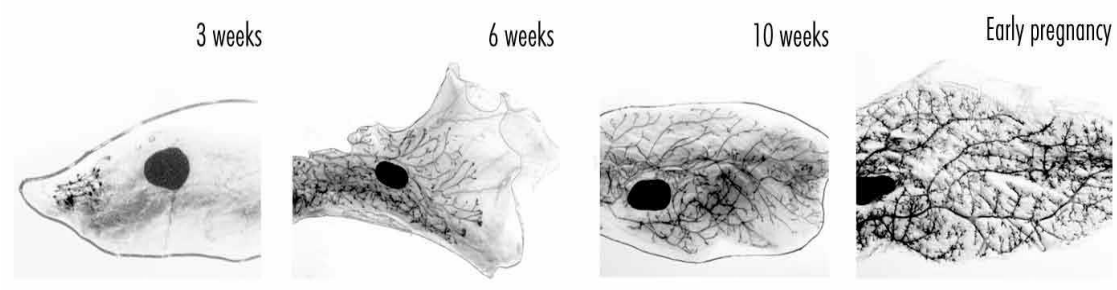

Figure 2: Mammary gland development in the mouse. Wholemount haematoxylin staining of the developing mammary gland highlighting the increasing complexity of the ductal tree in response to the secretion of hormones at puberty and pregnancy.

Breast Cancer

According to the Center for Disease Control and the National Cancer Institute, 220,097 women and 2,078 men were diagnosed with breast cancer in 2011. For women, over 200,000 new cases are predicted for 2014 and 1 in 8 women will be diagnosed with invasive breast cancer in her lifetime (Group, 2014). While many advances have been made in understanding and treating the disease, it remains a serious health threat to women with significant side effects from available treatments.

One advance that has helped to inform more personalized and targeted treatment is molecular subtyping. Transcriptional profiling studies were able to identify five molecular subtypes of breast cancer (Perou et al., 2000). Luminal A has a low proliferative index and expresses *estrogen* and *progesterone-receptors* and is not enriched for *human epidermal growth factor receptor-2*. This is the most common tumor type and in general responds well to treatment. Luminal B tumors are *estrogen* and *progesterone receptors* (+) and *human epidermal growth factor receptor-2* enriched or if *human epidermal growth factor receptor-2* negative, has a high proliferative index. Human epidermal growth factor receptor-2 type tumors are typically *estrogen* and *progesterone receptors* (–) and h*uman epidermal growth factor receptor-2* (+) but may be *human epidermal growth factor receptor-2* (-). These tumors have a high proliferative index, confer poorer prognosis than luminal tumors and a high percentage of these tumors contain *p53* mutations. Triple negative breast cancer, often referred to as basal, is *estrogen* and *progesterone receptors* (–) and *human epidermal growth factor*

receptor 2 (-) and the prognosis for women with this type of cancer is not as good as those with tumors of luminal origin due to its heterogeneity and paucity of therapeutic targets (Elsamany and Abdullah, 2014). Claudin-low tumors are triple negative breast cancers and are characterized by involvement of the immune response and the presence of a cancer stem cell signature, meaning the cells are more stem like (Romero et al.). The molecular subtypes describe large classes of tumors and not all tumors within each subtypes responds to treatment in the same way. In order to continue to refine and advance treatment, the subclasses within subtypes will need to be identified.

Bone morphogenetic proteins (BMPs) have been shown to play a role in breast cancer but results have been contradictory as in some studies BMP acted as a tumor suppressor (Buijs et al., 2011a; Chen et al., 2012) and in others as a tumor promoter (Owens et al., 2011). We are just beginning to understand that time of expression, which BMP ligand is expressed and the extracellular milieu are key determinants in the ultimate cellular response (Masuda et al., 2011; Rodriguez-Martinez et al., 2011; Takahashi et al., 2008)

BMP signaling and its regulation by TWSG1.

BMPs are members of the Transforming growth factor β superfamily and have diverse functions during development, including regulation of cell proliferation, migration, differentiation and apoptosis (Sieber et al., 2009). Binding of BMPs to their receptors activates both Smad-dependent and Smad independent pathways (Miyazawa et al., 2002). Classically, BMP binding leads to phosphorylation of cytoplasmic SMAD1/5/8 proteins and their subsequent binding with SMAD4. This leads to nuclear translocation where the complex acts as transcription factors to activate or repress target genes (Miyazawa et al., 2002; Wotton and Massague, 2001). BMP signaling is tightly regulated at many levels and can be modulated in the extracellular space by the secreted proteins Chordin, Noggin, and TWSG1 (Figure 3) among others (Table 1) (Gazzerro and Canalis, 2006).

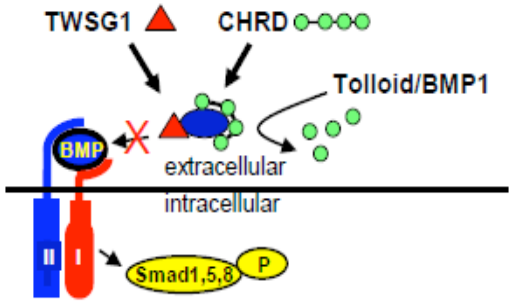

Figure 3: Schematic of Twisted gastrulation-1 with bone morphogenetic protein and Chordin in a tripartite complex.

TABLE 1: Selected BMP antagonists and their role in development.

Common Name	Gene Symbol	Human Chromosome Locus	Role in Development
Chordin	*Chrd*	3q27	Multiple roles in skeletal, ear, pharyngeal, cardiovascular development (Bachiller et al., 2000).
Chordin-like1	*Neuralin-1*	Xq22.1-q23	Secondary axis forms; Expressed in mesenchymal lineages (Nakayama et al., 2001)
Cerberus	*Cer1*	9p23–p22	Head-inducing ability in *Xenopus* (Bouwmeester et al., 1996), although apparently non-crucial in knockout *Mus musculus* studies (Bachiller et al., 2000).
Noggin	*Nog*	17q21–q22	Dorsoventral patterning, limb formation, neural tube morphogenesis (Brunet et al., 1998).
Twisted gastrulation	*Twsg1*	18p11.3	Dorsoventral patterning (Walsh et al.), salivary gland morphogenesis (Melnick et al., 2006) , postnatally viable in knockout mice, with intermittent skeletal complications. In *Xenopus*, reduced Tsg expression results in ventralized development and lack of forebrain (Wills et al., 2006).
Gremlin	*Grem1*	15q13–q15	Crucial for kidney formation (Michos et al., 2004); and limb outgrowth and digitation homozygous (Zuniga et al., 1999) null mouse is postnatal lethal.
Usag1	*Sostdc1*	7p21.1	Expressed in uterine tissues, although crucial for proper dental formation and numeration in mice (Simmons and Kennedy, 2002).
Sclerostin	*Sost*	17q11.2	Responsible for sclerosteosis; crucial for control of bone mass and outgrowth (Brunkow et al., 2001). Postnatally viable, although with increased mortality and morbidity.
Coco	*Dand5*	19p13.2	Left–right body symmetry (Walsh et al.).
DAN	*Nbl1*	4 D3; 4 70.0 cM	No abnormal phenotype associated with DAN null mouse.

TWSG1 inhibits BMP signaling by binding BMP ligands and preventing the ligand from binding to its receptor. TWSG1 can also promote BMP signaling as previously discussed. Currently, it is not known how BMP signaling is regulated in the mammary gland.

Very little is known about BMPs during mammary gland development beyond early embryonic development where BMP4 is required for placode specification (Hens et al., 2007). However, there are a few studies that suggest a role for BMPs in lumen formation (Montesano, 2007) and lactogenic differentiation (Perotti et al., 2012). Further elucidation of BMPs role in mammary gland development is hampered by the fact that BMP knockout mouse models tend to be embryonic lethal and tissue specific models may miss critical dose and timing requirements. *Twsg1* null mice are informative as BMPs are expressed in the right tissues at the right time but the dosage is modulated by the absence of this extracellular BMP binding protein.

Twsg1 Mouse Models

Of the *Twsg1* mouse models that have been generated skeletal and craniofacial defects were the most common phenotype reported (Gazzerro et al., 2006; Nosaka et al., 2003; Zakin and De Robertis, 2004). The model generated by our lab deleted the fourth exon by LoxP-flanking and in a C57Bl/6 syngenic background, midline craniofacial

defects characteristic of holoprosencephaly were seen (Petryk et al., 2004). While jaw defects, micrognathia to agnathia, were the most common, anterior truncation and cleft lip and palate were also observed. The craniofacial defects were not 100% penetrant with 44% percent of homozygous pups showing defects of the face and jaw. We also observed a high percentage of phenotypically normal animals born live to homozygous dams but not surviving beyond 48 hours. These pups were examined and found to have no gross anatomical malformations but curiously no milk spot was observed in the abdomen. To rule out subtle defects such as impaired sucking, these animals were fostered to wild type dams and wild type pups were fostered to *Twsg1*$^{-/-}$ dams. Milk spots were observed in all pups fostered to wild type dams regardless of pup's genotype and no milk spot was observed in pups fostered to *Twsg1* $^{-/-}$ dams (Figure 4). Homozygous pups fostered to wild type dams survived to adulthood while wild type pups fostered to *Twsg1*$^{-/-}$ dams died within 48 hours. These fostered wild type pups lacked a milk spot suggesting that *Twsg1*$^{-/-}$ dams have a mammary gland defect that prevents production and/or secretion of milk.

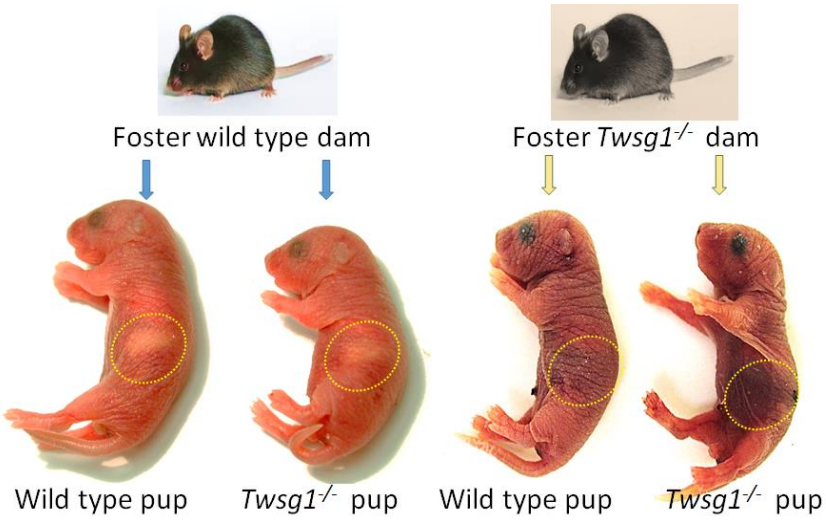

Figure 4: Milk spot observtion of pups fostered to wild type and *Twsg1*$^{-/-}$ dams.

Objective, rationale, summary

The objective of this thesis project has been to identify the molecular mechanism for *Twsg1* in mammary gland development and its role in breast cancer.

The rationale for these investigations lies in our understanding that developmental pathways reemerge in the development and progression of cancer. Therefore, we must first discover the developmental role for *Twsg1* in order to understand the consequences of misregulation during disease.

This thesis examines first the role of *Twsg1* in the development of the mammary gland in Chapter 2 and Chapter 3 explores *Twsg1* role in breast cancer with a focus on cell behavior.

Specific Aims:

Aim 1: Characterize the defect in *Twsg1*$^{-/-}$ mammary glands and identify molecular mechanisms.

Aim 2: Determine the role of *Twsg1* in breast cancer cell behavior.

Chapter 2: BMP-binding protein Twisted gastrulation is required in mammary gland epithelium for normal ductal elongation and myoepithelial compartmentalization

Introduction

BMPs, members of the Transforming growth factor β superfamily, are morphogens that play diverse roles during embryonic development and postnatal life (Wagner et al., 2010). BMPs have been implicated in regulating tissue homeostasis in physiological states as well as pathological ones, such as cancer, by mediating basic biological processes including cell proliferation, migration, differentiation, apoptosis, and epithelial-stromal interactions (Buijs et al., 2011b; Singh and Morris, 2010; Virtanen et al., 2011; Wang et al., 2011b). There is growing evidence that developmental programs that are important during embryonic development and adult homeostasis can be inappropriately reactivated during the initiation and progression of cancer (Bowman and Nusse, 2011; McEvoy et al., 2011; Wang et al., 2011c). Specifically, BMPs play important roles during embryonic mammary gland development and have been shown to be dysregulated in breast and other cancers often acting as a tumor suppressor or tumor promoter depending on dosage, time and tissue of expression (Bailey et al., 2007; Beppu et al., 2008; Cho et al., 2006; Liu et al., 2008). Given that developmental programs, such as BMP mediated processes, are co-opted or exploited by tumorigenic cells, it is

important to understand what roles BMPs play during normal mammary gland development and how their activity is regulated.

In both humans and mice, the mammary gland is a dynamic organ that responds and develops over the lifetime of the female. Embryonic development establishes the rudimentary ductal tree that during puberty, pregnancy and lactation undergoes complex morphological changes (Hens and Wysolmerski, 2005 and Hovey and Trott, 2004). As described earlier, the mouse, mammary gland development begins around embryonic day 10.5 when epithelial thickenings form placodes, which further thicken and invaginate as they respond to inductive signals from the underlying mesenchyme at around embryonic day 13.5. At approximately embryonic day 16.5, the primary bud epithelium proliferates and elongates into the developing fat pad forming a rudimentary tree. Development is arrested at this point until puberty. At puberty, terminal end buds form at the leading edge of the duct and are the site of active proliferation and apoptosis (Dulbecco et al., 1982 and Humphreys et al., 1996). The terminal end bud consists of bipotent cap cells that generate both cytokeratin-18 positive luminal and cytokeratin-14 positive myoepithelial cells. The latter remain in contact with the extracellular matrix while the luminal cells polarize with their apical aspect oriented towards the lumen. In the terminal end bud, proliferation creates excess body cells, which generate the mass of the duct as it elongates and a subset of these cells undergo apoptosis to form the lumen. Clearing of excess cells is complete and the mature ductal structure can be seen beginning at the shoulder of the

terminal end bud (Humphreys et al., 1996). A mature duct consists of a layer, one to two cells wide, of cytokeratin-18 positive luminal cells and a single cell layer of cytokeratin14 positive myoepithelial basal cells. Once the ducts reach the edges of the fat pad, terminal end buds are reabsorbed and elongation ceases. Concomitant with elongation, the ductal tree is further elaborated by a tightly regulated process of secondary and tertiary branching.

The dynamic changes seen in the mammary gland are regulated by morphogens (Jardé and Dale, 2012 and Schwertfeger, 2009), cytokines/growth factors (McNally and Martin, 2011 and Watson et al., 2011) and hormones (McNally and Martin, 2011). Integration and regulation of these signals is required for the proper establishment of the mammary gland anlagen and subsequent development through puberty and pregnancy. Past studies have suggested that BMP2 and 4 play a role during mammary gland development, including bud formation, epithelial mesenchymal communication, proliferation and lumen formation (Hens et al., 2007, Montesano, 2007, Phippard et al., 1996 and van Genderen, I. van Genderen et al. (1994). et al., 1994), but understanding of the regulation of BMP signaling during mammary gland development is limited.

The BMP specific intracellular signal transducers of BMPs are SMAD 1/5/8 proteins, which become phosphorylated upon binding of BMPs (BMP 2, 4, 6 or 7 among others) to their receptors (BMP receptor 1A, BMP receptor 1B and BMP receptor 2). This binding can be facilitated by crossveinless 2 (CV2) (Zhang et al., 2010) or other receptor

complex proteins. Phosphorylated pSMAD 1/5/8 binds SMAD4 and translocates to the nucleus where the complex acts as a transcription factor (Massague et al., 2005 and Miyazono et al., 2005). In the extracellular space, availability of BMPs for binding to their receptors is regulated by the secreted proteins Chordin , Chordin-like 1, Chordin-like 2, Noggin and TWSG1 among others (Balemans and Van Hul, 2002). While Chordin and Noggin are antagonists of BMP action, TWSG1 has a dual role, acting as either a BMP antagonist or as an agonist (Oelgeschlager et al., 2000 and Ross et al., 2001) which has been shown in *Danio rerio*, *Drosophila melanogaster* and *Xenopus laevis* (Little and Mullins, 2004, Oelgeschlager et al., 2000 and Shimmi et al. (2005). Chordin can be cleaved by the matrix metalloproteinase BMP1, also known as tolloid, which can release ligand into the extracellular space. Cleavage of Chordin by BMP1 is enhanced when TWSG1 participates in the complex of Chordin and BMP (Xie and Fisher, 2005).

Mice deficient for TWSG1 have a number of developmental defects, including craniofacial malformations (Billington et al., 2011b, MacKenzie et al., 2009 and Zakin and De Robertis, 2004), defects of the vertebrae, kidneys, thymus (Nosaka et al., 2003), and other organs that require branching morphogenesis such as salivary glands (Melnick et al., 2006). Given the role of TWSG1 in the development of branched organs, the goal of this study was to understand the role of TWSG1 during postnatal ductal maturation in the mammary gland and to determine how perturbations in BMP signaling may affect that maturation.

Materials and methods

Mice

Generation and genotyping of mice deficient for Twisted gastrulation 1 (C57BL/6 background) and heterozygous gene targeted *Twsg1*/Lac-Z knock-in mice have been previously reported (Gazzerro et al., 2006 and Petryk et al., 2004). Use and care of the mice in this study were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Inguinal mammary glands (#4) were harvested from female mice in early puberty (Nelson et al., 1990) (day of vaginal opening, about six weeks of age). Some mice were allowed to recover and the contralateral mammary gland was harvested at 10 weeks and stained as previously described (Li et al., 2002). Mammary glands from 3 wk old females were also collected. Mammary glands that were collected from 10 wk animals were staged for metestrus by vaginal observation and vaginal smears. Briefly, whole glands were fixed in 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) for 2 h on ice, defatted with acetone and stained with hematoxylin (Fischer Scientific, Waltham, MA), cleared with xylenes and the gland flattened between two slides for image capture. For whole-mount lacZ staining, fat pads containing the mammary glands were isolated, fixed in ice-cold 2% formaldehyde, 0.2% gluteraldehyde, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 (NP40) in PBS for 5 min, washed with 2 mM MgCl2 in PBS, and stained in

X-gal solution [0.1 M phosphate pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP40, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6] supplemented with 1 mg X-Gal (Promega) on a rotating wheel, at room temperature, in the dark and overnight. Following washing in PBS and refixing in 2% formaldehyde, 0.2% gluteraldehyde, the pads where photographed or dehydrated and processed for paraffin embedding and sectioning. The extent of fat pad colonization, secondary branching and terminal end bud area were calculated as previously described (Khialeeva et al., 2011, McCaffrey and Macara, 2009 and Richards et al., 2004). Briefly, the length and width of the ductal tree were measured as well as the length and width of the stromal fat pad. Areas were calculated for both, and a ductal tree:fat pad ratio was calculated. The extent of secondary branching was calculated by counting branch points within a 2×3-mm2 area within 1 mm of the lymph node and terminal end bud area was calculated by using the freehand tool in ImageJ (Rasband, 1997–2011) to trace the terminal end bud, diameter was also calculated using the straight line tool.

Immunohistochemistry

Female mice at the onset of puberty were injected intraperitoneally (30 ng/g bodyweight) with bromo-2′-deoxy-uridine (BrdU) (Roche, Indianapolis, IN) and sacrificed 3 h later. Inguinal mammary glands (#4) were harvested and fixed in 4% paraformaldehyde on ice for 2 h, embedded in paraffin and sectioned at 5 μM. Sections were deparaffinized in xylenes, rehydrated through a series of graded ethanols. For

pSMAD1/5/8, GATA-3, Epithelial cadherin, cytokeratin-18 and cytokeratin-14 staining, antigen retrieval was performed. Slides were placed in Antigen Unmasking Solution diluted to manufacturer's recommendation (Vector Laboratories, Burlington, CA) and microwaved for 20 min. Once cooled, slides were washed and then blocked in PBS containing 5% goat serum (Invitrogen, Carlsbad, CA) and 0.3% Triton X-100 (Fisher Scientific) for 60 min at room temperature. Sections were incubated with anti-pSMAD1/5/8 (Cell Signaling Technology Inc., Danvers, MA) 1:50, anti-GATA-3 (BD Pharmigen, San Diego, CA) 1:50, anti-E-Cadherin (Cell Signaling Technology Inc.) 1:100, anti-Keratin-14 (Covance, Princeton, NJ) 1:200, anti-Keratin-18 (TROMA-I) (Developmental Studies Hybridoma Bank, Iowa City, IA) 1:200 or anti-Progesterone receptor (Sigma) 1:50. Sections were developed either with Alexa Fluor (Invitrogen) or DyLight (AbCam, San Francisco, CA) species-appropriate fluorophore conjugated secondary antibodies. Fluorescence was visualized on a Zeiss 710 LSM Confocal Microscope using an Argon12 laser (excitation 488 nm), a HeNe laser (excitation 543 nm) a HeNe laser (excitation 633). Images were taken under identical conditions and post-acquisition manipulations were also identical.

Cell culture

HC11 mouse mammary cells (Danielson et al., 1984) (a gift from Dr. Schwertfeger), derived from pregnant BALB/c mouse mammary gland were cultured in RPMI 1640 medium (ATCC, Manassas, VA) supplemented with 10% fetal calf serum

(ATCC), 5 μg/ml insulin (Sigma) and 10 ng/ml epidermal growth factor (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO2 in air at 37 °C. Prior to BMP treatment cells were serum starved for 24 h, and then treated with either BSA or recombinant BMP7 50 ng/ml (R&D Systems).

Cell proliferation and apoptosis

BrdU incorporation was visualized using bromo-2′-deoxy-uridine Labeling and Detection Kit II (Roche) following manufacturer's instruction and double stained with anti-cytokeratin-18 (Developmental Studies Hybridoma Bank) 1:200. Five terminal end buds per section (n=5) from five mammary glands were image captured. The number of BrdU positive cells and the total number of cytokeratin-18 positive cells were counted by two independent scorers who were blinded to genotype and the total number of BrdU positive cells are presented as a fraction of total nuclei. Using the same method, apoptotic cells were visualized with Terminal Uridine Nick-End Labeling staining (Gavrieli et al., 1992) using a DeadEnd Fluorometric Terminal Uridine Nick-End Labeling System (Promega, Madison, WI) following manufacturer's instructions. The total number of Terminal Uridine Nick-End Labeling positive nuclei within a terminal end bud structure was expressed as a percentage of all nuclei within that structure.

cDNA generation, RT-PCR and qRT-PCR

Whole inguinal mammary glands (#4) were collected from wild type and *Twsg1*$^{-/-}$ virgin female mice at the onset of puberty, lymph node removed, and mammary

gland lysed in TRIzol reagent (Invitrogen). Furthermore, inguinal mammary glands were collected and lysed in TRIzol from wild type mice at 3 weeks, 10 weeks and early pregnancy (10.5 days past coitus). After extraction with TRIzol, RNA was isolated using RNeasy micro kit (Qiagen, Germantown, MD) following manufacturer's instructions. A similar procedure was followed to obtain total RNA from HC11 cells. Reverse transcription was carried out with the Thermoscript RT-PCR System (Invitrogen) priming with Oligo (dT)20, followed by RT and qRT-PCR (Q-PCR, MX3000p, Agilent, LaJolla, CA) for BMP pathway components. Primers for BMP pathway components have been previously published (Sun et al., 2010). Our approach, given the expected reduced epithelium within the *Twsg1*$^{-/-}$ gland, was to first calculate a ratio between GAPDH and cytokeratin-18 and then use this ratio to normalize the signal of the gene of interest. This allowed us to look both at relative whole gland expression as well as relative epithelial expression.

Western blotting

Whole inguinal mammary glands were collected from virgin, female mice at the onset of puberty, lymph node removed and lysed in modified RIPA buffer (250 µl of 140 mM NaCl, 0.4 mM Tris–HCl pH 8.0, 1% Glycerol, 1% NP40, 2% BSA with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche)). SDS-PAGE was used to separate proteins and separated proteins were transferred to PVDF membrane. The membranes (Invitrogen) were blocked with Odyssey blocking buffer (LI-COR, Lincoln,

NE, USA) containing 0.1% v/v Tween 20. Membranes were incubated overnight at 4 °C with anti-p-Smad1/5/8 (Cell Signaling Technology Inc.) 1:250, anti-Total SMAD (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100 and anti-GAPDH (ABCam, Cambridge, MA) 1:5000 antibodies and washed before incubation with species-appropriate fluorescent conjugated secondary antibodies for 1 h at room temperature. After washing to remove trace detergent, membranes were analyzed using an Odyssey Infrared Imaging System (LI-COR; Millennium Science, Surrey Hills, Australia) using the manufacturer's protocol. Relative pSMAD1/5/8 was calculated by first normalizing the signal intensity for total SMAD to GAPDH to control for loading. Then pSMAD1/5/8 signal was expressed as a percentage of the total SMAD pool. To detect GATA-3 membranes were incubated overnight at 4 °C with anti-GATA-3 antibody at 1:50 dilution (AbCam, San Francisco, CA), probed with anti-mouse HRP-conjugated secondary antibody (Cell Signaling Technology Inc.), and visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Mammary gland transplantation

Mammary glands from 3 wk old *Twsg1*$^{-/-}$ host females or wild type host females were cleared of endogenous epithelium as described (DeOme et al., 1959). Donor mammary tissue was collected from adult wild type (wild type) and *Twsg1*$^{-/-}$ mice and minced into small fragments. wild type and *Twsg1*$^{-/-}$ mammary fragments were transplanted into the cleared host fat pads, with each host (both wild type and *Twsg1*$^{-/-}$

mouse receiving a wild type transplant and a contralateral *Twsg1*$^{-/-}$ transplant. Host fat pads containing transplanted epithelium were removed and processed for wholemount haematoxylin staining as described above. Approximately 80% of all transplants were able to colonize and elongate into the host fat pad.

Statistical analyses

Gene expression levels were normalized to cytokeratin-18/GAPDH and significance calculated using a Student's t-test with significance set at $p<0.05$. pSMAD signal intensity was normalized to total SMAD which was first normalized to GAPDH. Significance was calculated using a Student's t-test with significance set at $p<0.05$.

Results

TWSG1 and other BMP signaling pathway components are present in the mammary gland during postnatal development

To determine the presence and timing of *Twsg1* expression during mammary gland postnatal development, LacZ staining of mammary glands from heterozygous mice with LacZ inserted into the *Twsg1* locus and RT-PCR for BMP pathway components was performed. *Twsg1* was detected by LacZ staining in the myoepithelium and in a subset of body cells within the canalizing terminal end bud (Figure 5A and B) at 6 weeks. In the mature gland, *Twsg1* was expressed throughout the ductal tree and was primarily expressed in the myoepithelium (Figure 5C and D). Given that TWSG1 modulates BMP signaling, components of the BMP pathway were assessed by RT-PCR across

developmentally relevant time points (Figure 5E). mRNA for *BMP1, BMP2, BMP6, BMP7, BMP receptor-1a, BMP receptor-2,* and *Smad4* were expressed across all time points. The Type1 receptor *BMP receptor-1b* was barely detectable. *BMP4* was detected at 10 weeks and at pregnancy. Among genes encoding BMP-binding proteins, *Twsg1* mRNA was expressed at all time points while others were more temporally expressed. *Chordin* was undetectable at 6 weeks and barely detectable at other stages, while *Chordin-like 1* was strongly expressed at each time point. *Noggin* was expressed only during pregnancy while *Chordin-like 2* was undetectable (Figure 1). *Crossveinless 2* (an extracellular BMP binding protein) mRNA was not detected during pregnancy, but was present at other time points.

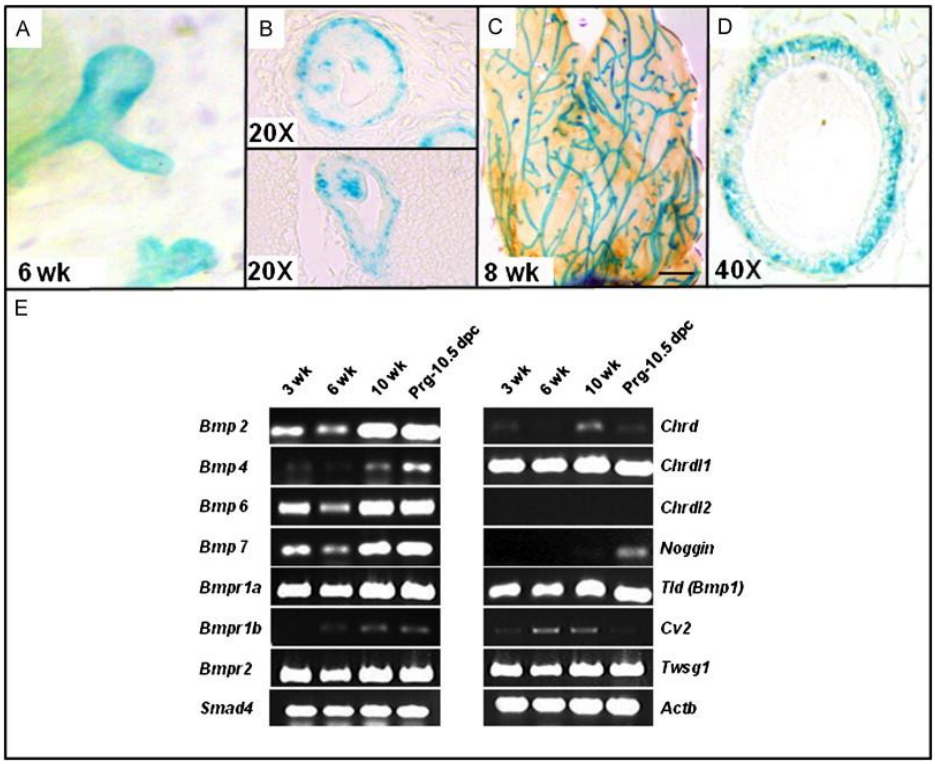

Figure 5: *Twsg1* and BMP pathway components are expressed in the mammary gland at important postnatal developmental time points. (A–D) LacZ staining of whole mount (A and C) and sectioned (B and D) mammary glands isolated from heterozygous 6 and 8 wk old virgin female carrying *LacZ* reporter gene in the *Twsg1* locus. (A) *Twsg1* is present in ducts and terminal end buds at 6 weeks (B) and in a subset of body cells in the canalizing terminal end bud. (C) *Twsg1* expression is present in the mature ducts (2 mm scale bar). (D) This expression is observed primarily in the myoepithelium at 8 weeks. (E) RT-PCR showing temporal expression of BMP pathway members in mammary glands at different postnatal developmental time points.*Actb*, beta-actin, *Chrd*, chordin; *Chrdl1*, chordin-like 1; *Chrdl2*, chordin-like 2; *Cv2*, crossveinless 2.

TWSG1 is required for timely ductal elongation and fat pad colonization

Mammary glands from wild type and *Twsg1*$^{-/-}$ female mice were examined at 3, 6 and 10 weeks. The ductal trees were comparable at 3 weeks with the establishment of a rudimentary tree (Figure 6A and D) although terminal end buds appeared atypical in the *Twsg1*$^{-/-}$ mammary gland (Figure 6G). At 3 weeks, terminal end buds were smaller than wild type and in some cases it appeared as if terminal end buds were not established. At 6

weeks, the wild type ducts had elongated well past the lymph node while the *Twsg1*$^{-/-}$ ducts showed little elongation. In most cases, the ducts did not reach or just reached the lymph node (Figure 6B and E). The terminal end buds in the *Twsg1*$^{-/-}$ were larger than wild type terminal end buds at this time point (Figure 6H). At 10 weeks, the wild type ducts had completely elongated, were well branched and the terminal end buds had reabsorbed. In *Twsg1*$^{-/-}$ ducts, terminal end buds were still present and the ductal tree was not as robustly branched (Figure 6C and F). At 10 weeks, the wild type terminal end buds had reabsorbed while terminal end buds were still found in the *Twsg1*$^{-/-}$ mammary gland (Figure 6I). A reduction in secondary branching in *Twsg1*$^{-/-}$ mammary glands at 10 weeks was also observed. The most striking difference between wild type and *Twsg1*$^{-/-}$ ductal morphogenesis was found at 6 weeks and this stage was assessed further.

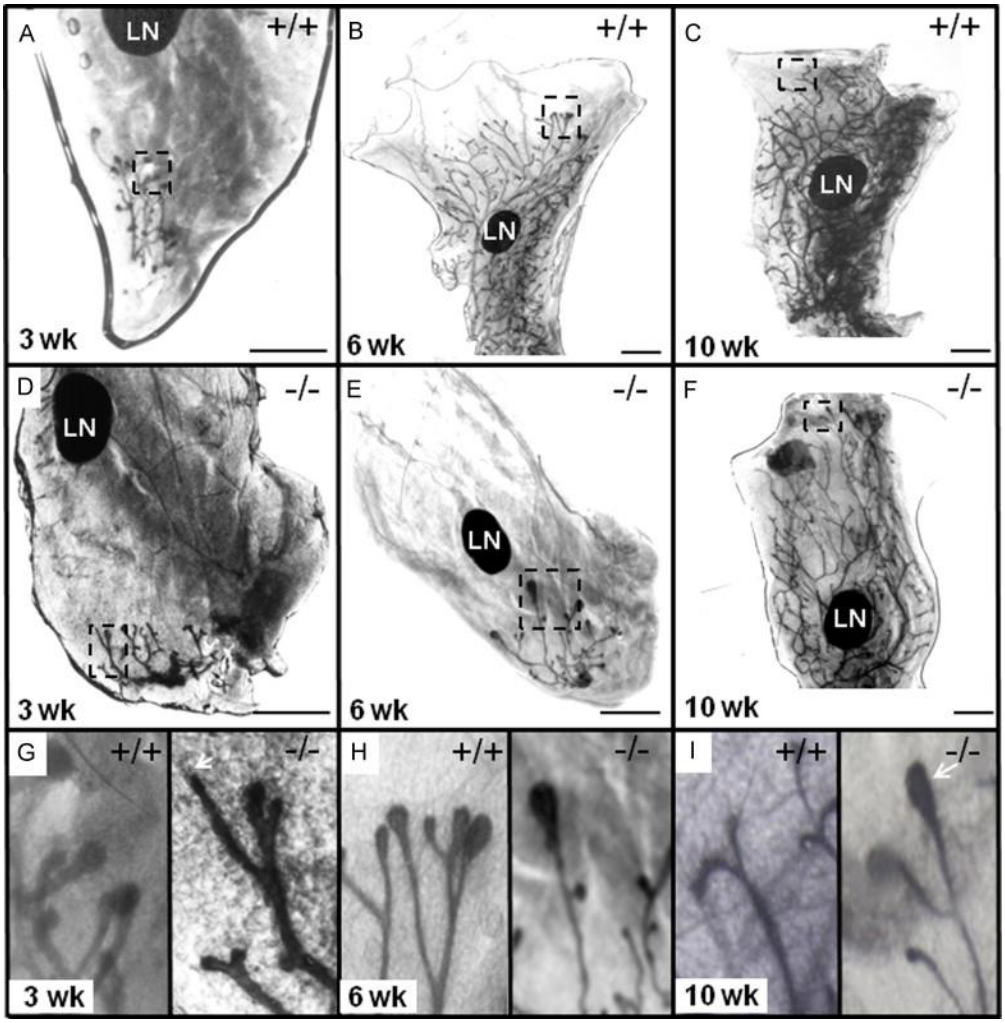

Figure 6: Loss of Twisted gastrulation 1 results in a delay of ductal elongation
(A, B, C) Whole mount mammary gland from 3 week, 6 week and 10 week old wild type (wild type) virgin female mouse. **(D, E, F)** Whole mount mammary gland from 3 week, 6 week and 10 week old *Twsg1*$^{-/-}$ virgin female mouse. **(G, H, I)** Distal ends of ductal tree from the mammary gland of 3 week, 6 week and 10 week wild type and *Twsg1*$^{-/-}$ virgin female mouse. Arrow indicates poorly formed terminal end bud (2G) and terminal end buds still present at 10 weeks (2I) in the *Twsg1*$^{-/-}$ mammary gland. Magnified distal ends are boxed. LN, lymph node. Scale bar 2 mm.

Mammary glands from *Twsg1*$^{-/-}$ female mice were examined on the first day of vaginal opening, approximately 6 weeks of age. Mammary glands from wild type animals contained ducts that extended past the lymph node and were robustly branched. In

contrast, *Twsg1*$^{-/-}$ mice showed a significant decrease in fat pad colonization (Figure 7A) with 38% of fat pad being occupied by the ductal network in *Twsg1*$^{-/-}$ mammary glands (n=5) compared to 64% in wild type mammary glands (n=5, p=0.0032) at 6 weeks of age. In addition, secondary branching was significantly reduced in mammary glands from 6 wk old virgin *Twsg1*$^{-/-}$ animals compared to wild type (Figure 7B) and terminal end buds were significantly larger in *Twsg1*$^{-/-}$ ducts (Figure 7C).

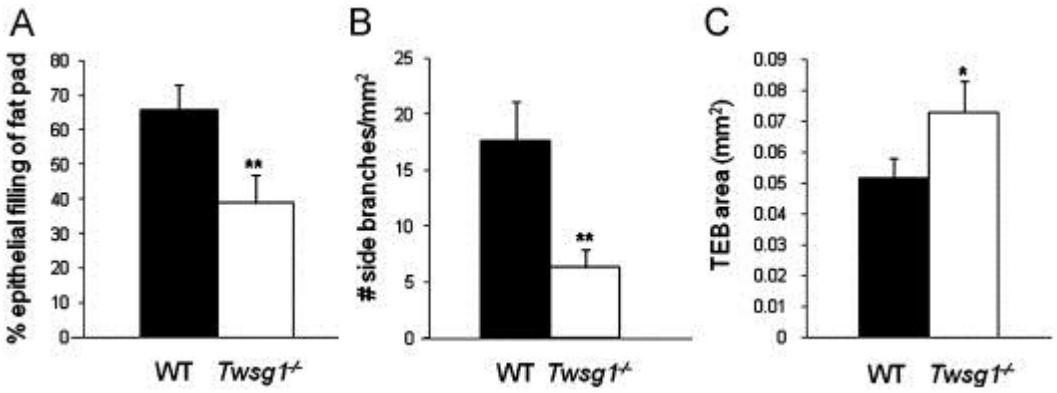

Figure 7: Loss of Twisted gastrulation 1 results in impaired ductal elongation, reduced secondary branching and enlarged terminal end buds. (A) Percentage of fat pad colonization by the ductal tree in wild type and *Twsg1*$^{-/-}$ virgin female mouse. (B) Side branching is significantly reduced in *Twsg1*$^{-/-}$ mammary gland. (C) terminal end buds are significantly larger in *Twsg1*$^{-/-}$ mammary glands at 6 weeks. ***p*<0.001

Delay in elongation is intrinsic to the mammary gland

To determine whether the delay in ductal elongation and fat pad colonization was intrinsic to the mammary gland rather than secondary to other factors, for example alterations in hormonal milieu, we performed transplantation experiments. We transplanted *Twsg1*$^{-/-}$ epithelium into the fat pads, cleared of endogenous epithelium, of 3 week old wild type animals. The mammary glands were analyzed 3 weeks later and there was either a significant delay (n=2) or no measurable outgrowth (n=1) of *Twsg1*$^{-/-}$

mammary gland tissue when compared to contralaterally transplanted wild type tissue (Figure 8 A and B). Conversely, wild type mammary gland tissue transplanted into the cleared fat pad of *Twsg1*$^{-/-}$ had outgrowth that was similar to wild type tissue transplanted into wild type fat pad (data not shown). The terminal end buds in the *Twsg1*$^{-/-}$ transplanted tissue were similar in morphology to terminal end buds in 3 week *Twsg1*$^{-/-}$ mammary glands (Figure 8 C and D).

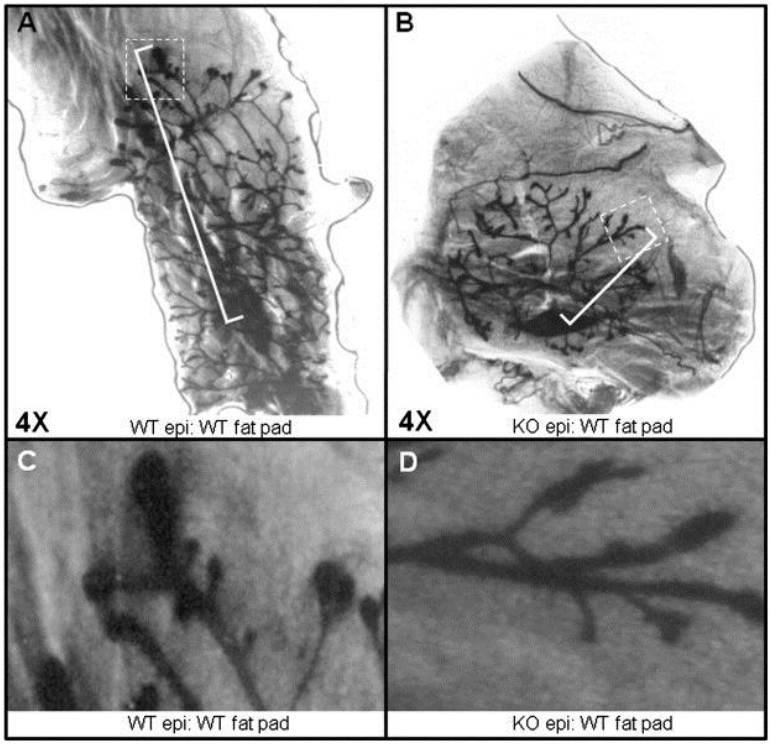

Figure 8: Elongation defect is intrinsic to the mammary gland. (A) wild type epithelium transplanted into cleared fat pad of 3 week old wild type mouse shows adequate elongation (*n*=3) as measured from center of transplanted epithelium to distal end of longest primary duct. Longest primary duct is bracketed and farthest distal end is boxed. (B) *Twsg1*$^{-/-}$ epithelium transplanted into cleared fat pad of 3 week old wild type mouse shows impaired elongation (*n*=3) as measured from center of transplanted epithelium to distal end of longest primary duct. Longest primary duct is bracketed and farthest distal end is boxed. (C) Magnified view of transplanted wild type distal ends. (D) Magnified view of transplanted *Twsg1*$^{-/-}$ distal ends.

BMP signaling is reduced in the mammary gland epithelium

To further investigate BMP signaling within the *Twsg1*$^{-/-}$ mammary gland, a western blot was performed to detect pSMAD1/5/8. BMP signaling via pSMAD was significantly reduced in the *Twsg1*$^{-/-}$ mammary gland (Figure 9A). Co-localization studies demonstrated that in the wild type mammary gland pSMAD1/5/8 was localized to nuclei of cells within the terminal end bud and pSMAD1/5/8 signal could not be detected in the *Twsg1*$^{-/-}$ terminal end bud (Figure 9B). We also observed that BMP downstream targets, *Msx1*, *Msx2* and *Gata-3*, were downregulated in *Twsg1*$^{-/-}$ mammary gland (Figure 9C).

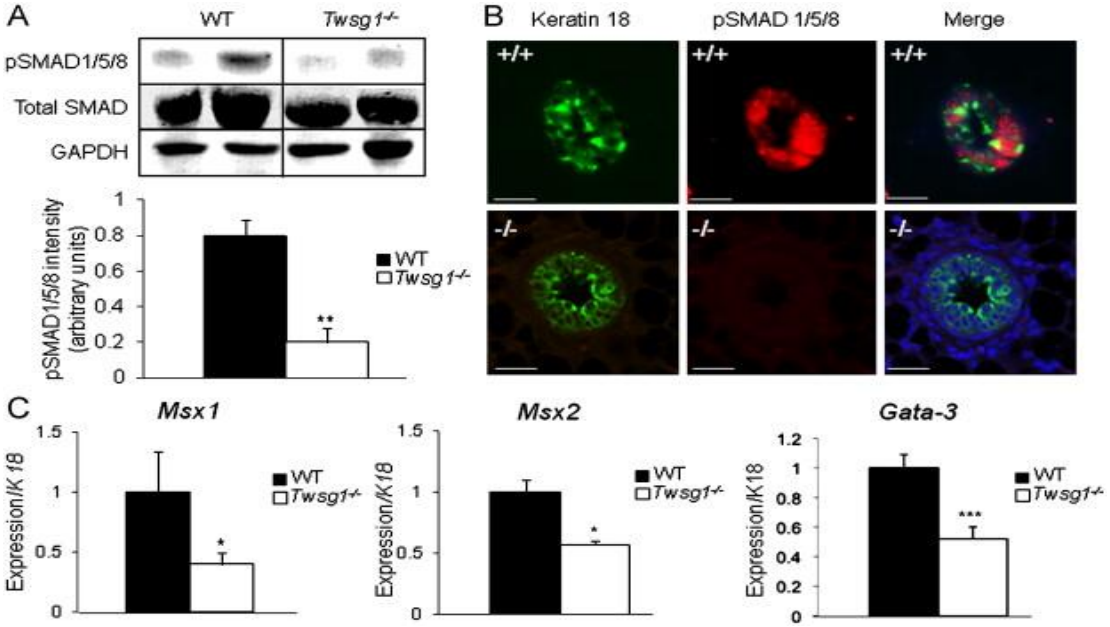

Figure 9: BMP signaling is reduced in mammary glands from *Twsg1*$^{-/-}$ animals. (A) Western blot, showing two representative samples per group, detecting the levels of total SMAD, pSMAD1/5/8 and GAPDH in mammary gland lysates from wild type and *Twsg1*$^{-/-}$ 6 week old virgins. Total SMAD was normalized to GAPDH and a ratio of SMAD to pSMAD1/5/8 was calculated; $p<0.002$. (B) Immunofluorescence staining for epithelial marker keratin 18 (green) and pSMAD1/5/8 (red). pSMAD1/5/8 is absent in *Twsg1*$^{-/-}$ mammary glands. (C)-PCR for BMP downstream targets, *Msx1*, *Msx2 and Gata3*, shows reduced expression in *Twsg1*$^{-/-}$ mammary glands. Scale bar 2 um.

TWSG1 mediates lumen formation and luminal identity

Whole mount light microscopy of *Twsg1*$^{-/-}$ mammary glands showed a density of staining in the duct that was suggestive of a difference in cell density between wild type and *Twsg1*$^{-/-}$ mammary glands. To investigate this further we sectioned mammary glands from 6 week old virgin females and stained with cytokeratin-18, cytokeratin-14 and Trichrome and assessed mature ducts. Wild type mammary glands at 6 weeks had mature ducts with a clear, well-defined lumen (Figure 10A–C, G–I, M and N) with no cells visible within the lumen. Conversely, the *Twsg1*$^{-/-}$ mammary glands contained a mix of ducts containing cell islets (Figure 10D–F), completely occluded ducts (Figure 10J–L) as well as single cells shed into the lumen (Figure 10Q and R) and clear lumens. Approximately 60% of the mature ducts in the *Twsg1*$^{-/-}$ mammary gland had either complete or partial occlusion. When occluded ducts were stained for cytokeratin-14, the cells within the lumens contained an organized structure that consisted of a secondary cytokeratin-14 positive compartment surrounding a mass of cytokeratin-14 negative cells (Figure 10D–F). This "duct within a duct" structure was only observed in *Twsg1*$^{-/-}$ mammary glands. Wild type, mature ducts consisted of a clearly defined luminal compartment that was cytokeratin 18 positive (Figure 10G–I). Using a blood vessel landmark, the luminal compartments of *Twsg1*$^{-/-}$ ducts were further examined. In many cases, the duct remained occluded until out of the plane of sectioning and not all cells within the occlusion were cytokeratin 18 positive (arrows) (Figure 10J–L). To look more closely at *Twsg1*$^{-/-}$ myoepithelial compartment, cytokeratin 14 stained sections were

interrogated for the presence of basal body cells, which are cytokeratin-14 positive cells within the luminal compartment. In wild type ducts, no basal body cells were observed (Figure 10M and N), consistent with previous reports (Mailleux et al., 2007). Within *Twsg1*$^{-/-}$ mammary glands ~60% of the ducts observed contained cytokeratin-14 positive basal body cells. To further characterize the cells within the luminal compartment ducts were stained for smooth muscle actin and a similar distribution of smooth muscle actin positive cells were seen in the *Twsg1*$^{-/-}$ mammary gland (data not shown). Additionally, we observed cell shedding in *Twsg1*$^{-/-}$ but not wild type mature ducts. Usually these were single or small groups of cells within the lumens of mature ducts and these shed cells were cytokeratin=14 positive (Figure 10Q and R).

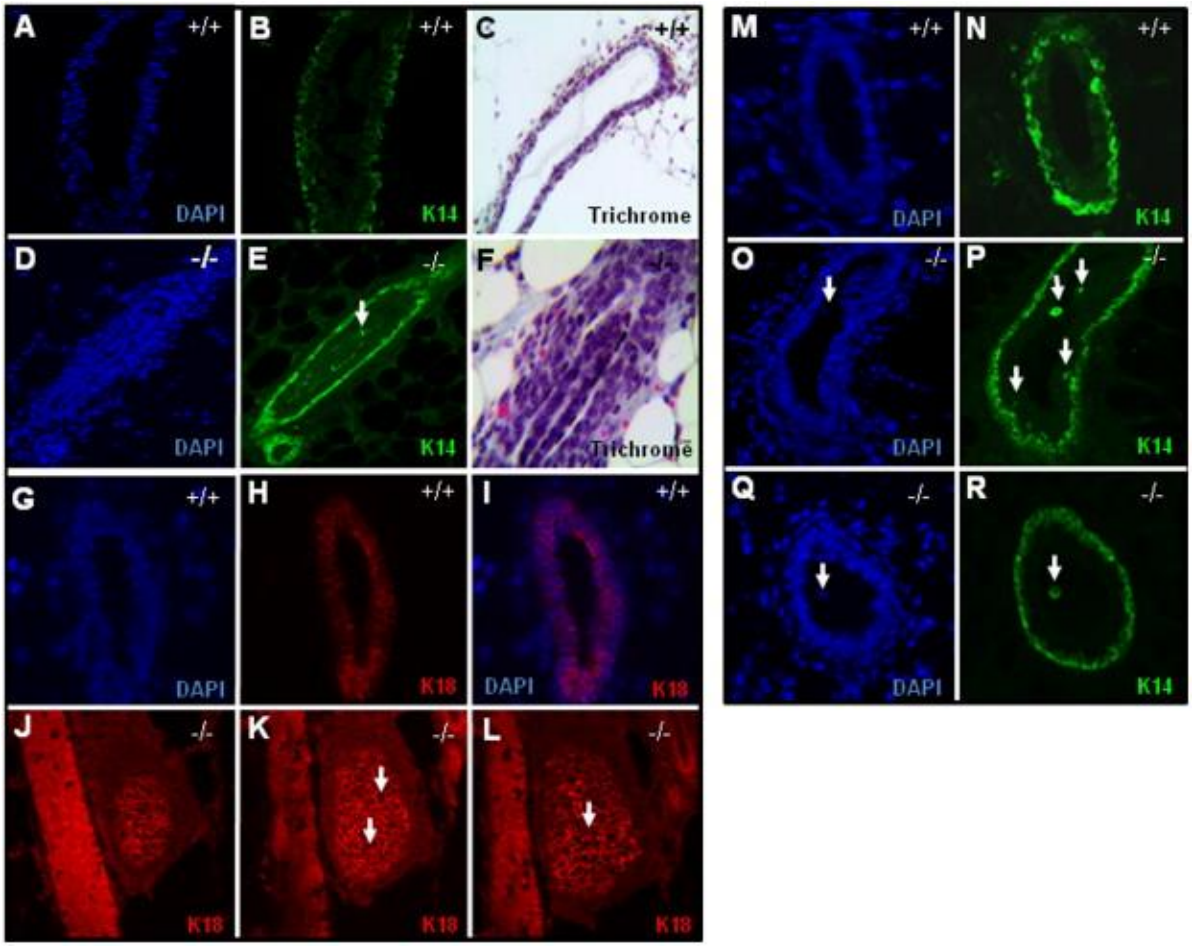

Figure 10: *Twsg1*$^{-/-}$ mammary glands have abnormal epithelial organization, occluded lumens and cell shedding at puberty. (A–C) wild type mammary glands (D–F) *Twsg1*$^{-/-}$ mammary glands stained with cytokeratin-14 have occluded lumens, which can be organized with a secondary myoepithelial compartment surrounding a mass of cytokeratin-14 negative cells. (C and F) Trichrome staining reveals cleared lumens of wild type ducts and an organized cell mass within some lumens of the *Twsg1*$^{-/-}$ maturing duct. (G–I) wild type ducts with a well formed lumen. (J–L) Using a blood vessel to mark the location within the mammary gland, serial sections of an occluded *Twsg1*$^{-/-}$ duct were stained for cytokeratin-18. Not all the cells that occlude the lumen are cytokeratin-18 positive (arrows). (M and N) cytokeratin-14 stained wild type mature ducts showing cytokeratin-14 positive cells in the basal compartment, but not the luminal compartment of the epithelium at puberty. (O and P) cytokeratin-14 positive cells are found in the luminal compartment of *Twsg1*$^{-/-}$ mature ducts (arrow). (Q and R) Cell shedding in *Twsg1*$^{-/-}$ducts. Cells shed into the lumen were preferentially cytokeratin-14 positive. All images were taken at 20×.

Apoptotic defect may lead to accumulation of cells and poor lumen formation in *Twsg1* mutant ducts

The terminal end buds of mammary glands from *Twsg1*$^{-/-}$ animals are larger than wild type controls suggesting hyperplasia. To assess this further we counted the number of cells within terminal end bud structures (five animals, five sections per animal) in wild type and *Twsg1*$^{-/-}$ mammary glands. There was a significant increase in the number of cytokeratin-18 positive cells within the *Twsg1*$^{-/-}$ terminal end buds (Figure 11A). To determine if this was due to an increase in proliferation or a decrease in apoptosis we stained for Bromodeoxyuridine and performed a Terminal deoxynucleotidyl transferase assay. Bromodeoxyuridine incorporation revealed no significant difference in proliferation between wild type and *Twsg1*$^{-/-}$ terminal end buds (Figure 11B and D). To determine if known regulators of proliferation are changed in our model, we assessed the expression of insulin-like growth factor 1, amphiregulin and estrogen receptor alpha by Q-PCR. There were no significant differences detected (data not shown). Furthermore, amphiregulin and progesterone receptor were visualized by immunofluorescence and no differences in distribution were observed (data not shown). However, there was a marked decrease of nearly four-fold in apoptosis within the *Twsg1*$^{-/-}$ terminal end buds (Figure 11C and D).

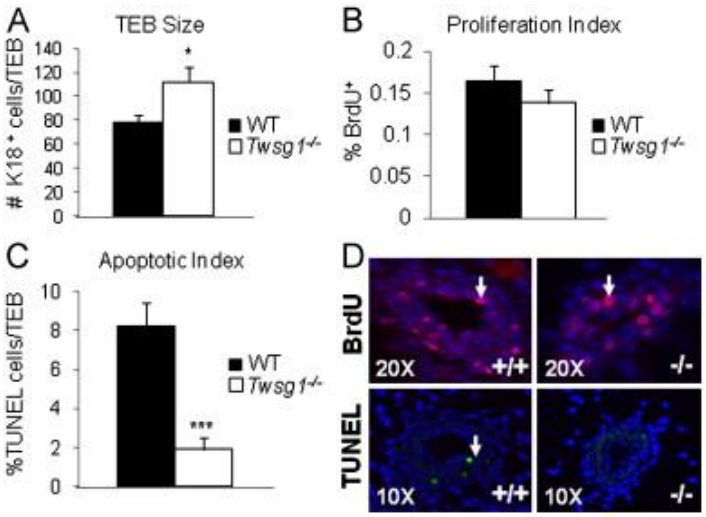

Figure 11: *Twsg1*$^{-/-}$ terminal end buds show an increase in luminal epithelial (cytokeratin-18) cell number and a decrease in apoptosis. (A) cytokeratin-18 positive cells were counted within terminal end buds and compared between wild type and *Twsg1*$^{-/-}$. A significant increase in cell number was observed in *Twsg1*$^{-/-}$ compared to wild type. (B) Bromodeoxyuridine positive cells were counted within the same compartment and no significant change in proliferation was observed. (C) Terminal deoxynucleotidyl transferase staining revealed a significant decrease in the number of apoptotic cells within the terminal end bud of *Twsg1*$^{-/-}$ mammary glands. (D) Representative images for Bromodeoxyuridine (pink), cytokeratin-18 (green) and Terminal deoxynucleotidyl transferase (green) staining. *p<0.05, ***p<0.001.

TWSG1 plays a role in restricting cytokeratin-14 positive cells to the basal compartment

Gata-3 is required for luminal identity in the mammary gland and is a downstream target of BMP signaling in various tissues (Bonilla-Claudio et al., 2012). In wild type terminal end buds (Figure 12A–D) and mature ducts (Figure 12I–L) almost all luminal cells expressed GATA-3. In contrast, *Twsg1*$^{-/-}$ mammary glands consisted of terminal end buds (Figure 12E–H) and mature ducts (Figure 12M–P) that were a mosaic of GATA-3 positive and GATA-3 negative cells. In *Twsg1*$^{-/-}$ mammary glands, single cells that were shed into the lumen were GATA-3 negative (Figure 12P). To confirm that

BMPs could induce *Gata-3* expression in mammary epithelial cells, HC11 cells were treated with BMP7 50 ng/ml and *Gata-3* mRNA expression measured by Q-PCR. BMP7 induced *Gata-3* expression over two-fold (p=0.0004) (Figure 13A). We also assessed *Gata-3* expression and protein content and found that *Gata-3* expression (Figure 13B) as well as protein level (Figure 13C) were reduced in *Twsg1*$^{-/-}$ mammary glands.

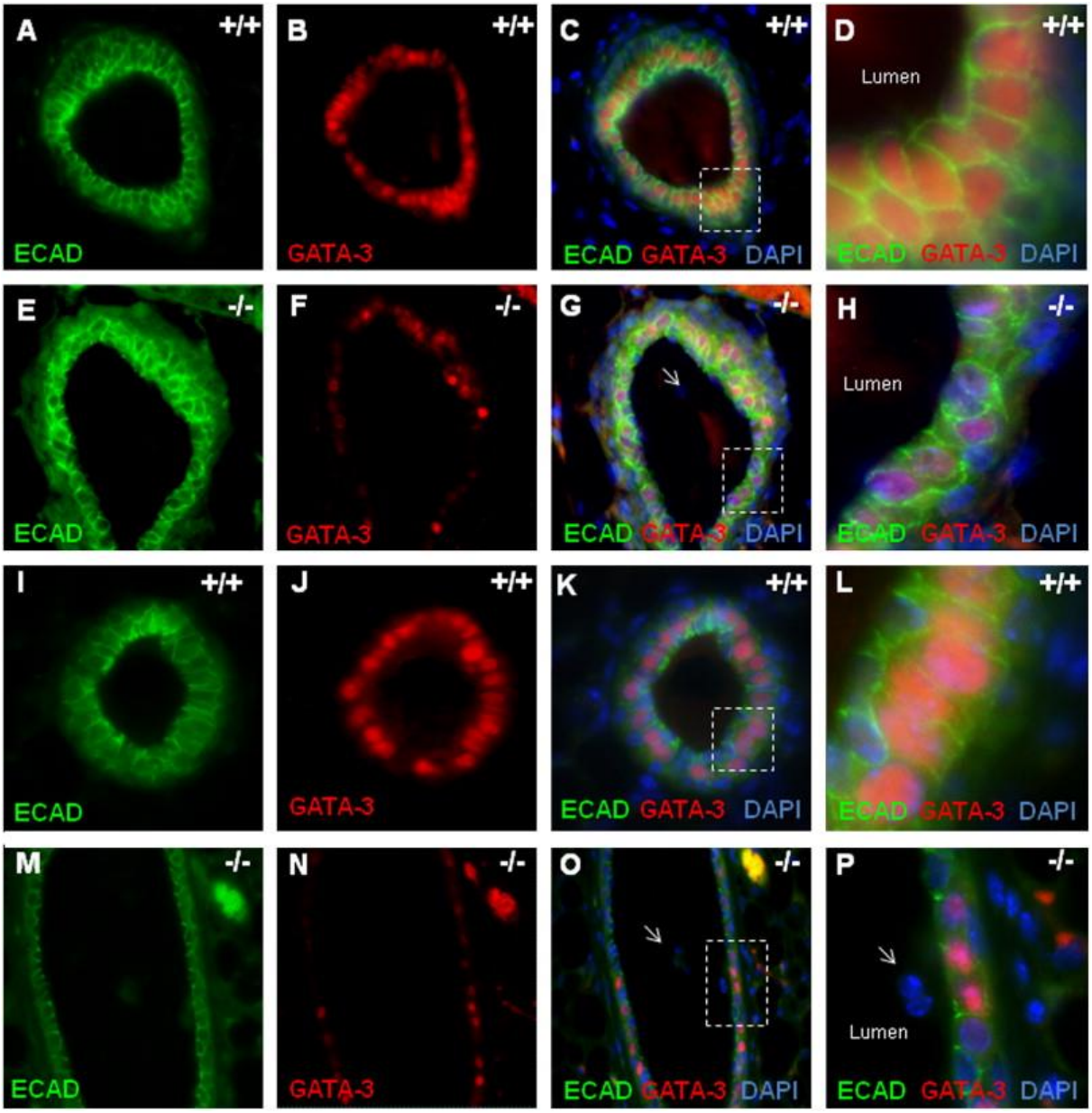

Figure 12: GATA-3, required for luminal identity, is reduced in *Twsg1*$^{-/-}$ mammary glands. GATA-3 expression is reduced in *Twsg1*$^{-/-}$ mammary glands. ECAD was used to mark luminal epithelial cells and most ECAD positive cells in the wild type terminal end bud express GATA-3 (A–D) and mature duct (I–L) while the *Twsg1*$^{-/-}$ terminal end bud (E–H) and mature duct (I–L) consist of a mosaic of GATA-3 positive and GATA-3 negative luminal cells. Most cells shed into the lumen are GATA-3 negative (P). All images were taken at 40×. Boxes in C, G, K, and O correspond to the magnified images in D, H, L, and P, respectively.

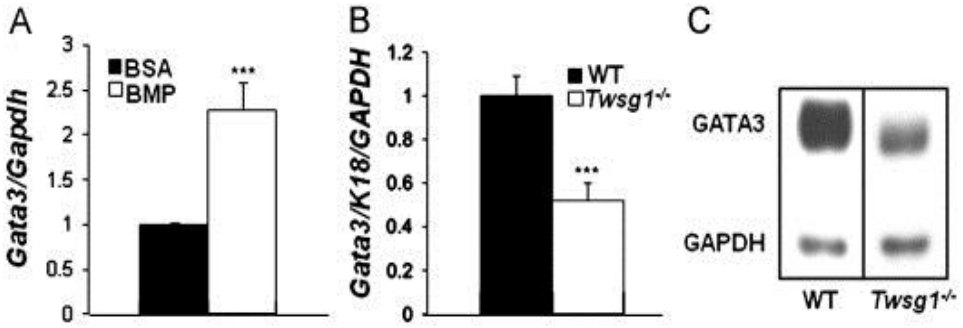

Figure 13: GATA-3, required for luminal identity, is reduced in *Twsg1*$^{-/-}$ mammary glands. (A) *Gata-3* expression is significantly upregulated in HC11 cells that have been treated with BMP7 (50 ng/ml). (B) *Gata-3* expression is significantly downregulated, in *Twsg1*$^{-/-}$mammary glands from 6 week old virgin mice. (C) There is reduced GATA-3 protein in *Twsg1*$^{-/-}$ mammary glands (*n*=3) compared to wild type (*n*=3).

Discussion

TWSG1 is a positive regulator of BMP signaling during postnatal mammary gland development. Studies in *Drosophila melanogaster, Danio rerio, Xenopus laevis,* and *Mus musculus* have shown that TWSG1 is a highly conserved extracellular modulator of BMP signaling with important roles during embryonic development (Ross et al., 2001, Scott et al., 2001 and Zusman and Wieschaus, 1985). Recent studies have demonstrated that TWSG1 continues to be an important BMP regulator in adult mammalian tissues, including bone homeostasis (Sotillo Rodriguez et al., 2009), regeneration following ischemic kidney injury (Larman et al., 2009), immune responses

(Tsalavos et al., 2011), and, as this study shows, postnatal mammary gland ductal maturation.

It is known that TWSG1 interacts with BMP2, BMP4, and BMP7 (Billington et al., 2011a, Blitz et al., 2003, Chang et al., 2001, Ross et al., 2001 and Scott et al., 2001), either directly or by forming complexes with Chordin (Larrain et al., 2001). While in *Xenopus laevis* and *Denio rario* TWSG1 can act as a BMP antagonist as well as an agonist (Blitz et al., 2003, Ross et al., 2001 and Xie and Fisher, 2005), the latter function has not been directly demonstrated in mammals. In this study, BMP signaling is reduced in the murine mammary gland in the absence of TWSG1, suggesting an agonist role for TWSG1 in the postnatal mammary gland. Chordin was not detected in the mammary gland at 6 weeks, suggesting that Chordin-independent mechanisms may play a role (Xie and Fisher, 2005) A potential binding partner is Chordin-like-1, which is highly expressed in the developing mammary gland and has been shown to bind BMPs and TWSG1. Intriguingly, in cells of the proximal tubule of the kidney, Chordin-like-1 amplifies BMP4 signaling in the presence of TWSG1 (Larman et al., 2009).

Reduced expression of the BMP targets *Msx1, Msx2, Gata-3*, and decreased levels of pSMAD1/5/8 all indicate a reduction of BMP signaling in the *Twsg1*$^{-/-}$ mammary gland. Mutations in *Msx1* and *Msx2* result in impaired mammary gland development. Mammary glands of *Msx2*-deficient mice arrest at the mammary sprout stage while the epithelium of the *Msx1/Msx2* double-deficient mice fails to form a bud with subsequent

regression of the mammary gland anlagen (Satokata et al., 2000). *BMP2* and *BMP4* mutants are embryonic lethal (Lawson et al., 1999 and Zhang and Bradley, 1996) and no studies have specifically addressed the effects of deletion of these genes in the postnatal mammary gland. Thus, the *Twsg1*$^{-/-}$ mouse provides a model in which to evaluate the consequences of perturbated BMP signaling during postnatal ductal maturation.

TWSG1 is essential for postnatal ductal maturation in the murine mammary gland.

Postnatal mammary gland development is orchestrated by numerous hormones, growth factors, cytokines, and transcription factors including MSX1, MSX2 and GATA-3, which regulate proliferation, apoptosis, and differentiation (McNally and Martin, 2011). Although BMPs are known to regulate proliferation through Msx1 (Zhang et al., 2002) and the expression of *Msx1* was decreased in the *Twsg1*$^{-/-}$ mammary gland, proliferation was not significantly altered suggesting that other pathways are involved in regulating proliferation in the elongating duct. In contrast, apoptosis was reduced in *Twsg1*$^{-/-}$ mammary gland, indicating a role for BMP-induced apoptosis in lumen formation. Furthermore, *Twsg1* is expressed in a subset of body cells within the terminal end bud making it available for regulation of BMP signaling within the canalizing terminal end bud. The classical mediator of the pro-apoptotic effects of BMPs is *Msx2* (Graham et al., 1996). Indeed, reduced *Msx2* expression was seen in the *Twsg1*$^{-/-}$ mammary gland. The reduced apoptosis in the terminal end bud could explain the observation that 60% of the mature ducts in the *Twsg1*$^{-/-}$ mammary gland were occluded

or had cell islets present in the lumen. It has been shown that disrupted apoptosis can be accompanied by lumen filling and cell shedding in the mammary gland (Mailleux et al., 2007). However, it is also possible that cells shed into the lumen repopulate the lumen with a mass of cells that is later cleared by some unknown mechanism.

GATA-3 is directly induced by BMP signaling during facial skeletal development (Bonilla-Claudio et al., 2012), preplacodal ectoderm specification (Kwon et al., 2010), and hair follicle morphogenesis (Kobielak et al., 2003). In the mammary gland, GATA-3 deficiency results in impaired placode formation, elongation defect as a result of the failure of terminal end buds to stably form, and a reduction in side branching (Asselin-Labat et al., 2007). Additionally, GATA-3 can induce apoptosis in a luminal tumor model (Kouros-Mehr et al., 2008) and upregulate pro-apoptotic genes such as caspase-14 (Asselin-Labat et al., 2011). In mice with mammary gland specific GATA-3 deficiency lumens are irregular and cell shedding is observed (Asselin-Labat et al., 2007). In the *Twsg1*$^{-/-}$ mammary gland, loss of *Twsg1* reduces BMP signaling perhaps by reducing BMP availability. As a result, not all cells reach the BMP signaling threshold required for *Twsg1*$^{-/-}$ induction and subsequent adoption of a luminal cell fate. The cells that fail to induce *Gata-3* will remain in the luminal compartment or be shed into the lumen. It is possible that this mosaic of cell types in the advancing duct inhibits elongation but as cells are lost into the lumen cell:cell contacts are reestablished and elongation can again proceed in the absence of *Twsg1*$^{-/-}$.

We also observed a decrease in secondary branching in *Twsg1*$^{-/-}$ mammary gland. As the ducts invade into the fat pad, secondary branching occurs in areas where there is low Transforming growth factor-β expression (Pierce et al., 1993) and high *Msx2* expression (Satoh et al., 2004). A recent study demonstrated that BMP2/4 can induce branching in the mammary gland via *Msx2* upregulation (Fleming et al.). When *Twsg1* is deleted, some cells receive adequate BMP while others do not and in those regions with reduced BMP, *Msx2* expression is also reduced and branching is not initiated, thus leading to an overall reduction in secondary branching in the *Twsg1*$^{-/-}$ mammary gland.

Contribution of TWSG1 to maintaining basal vs. luminal epithelial identity

The mammary gland epithelium is composed of the basal myoepithelial cells and polarized luminal epithelial cells. It is thought that the different cell populations arise from multipotent progenitors, or mammary stem cells (Stingl et al., 1998). Interestingly, in *Twsg1*$^{-/-}$ mammary gland, cytokeratin-14 positive cells are present within the luminal compartment among cytokeratin-18 positive cells. This suggests that BMP signaling plays a role in establishing and/or maintaining epithelial identity as opposed to myoepithelial, for example through GATA-3 (Metallo et al., 2008 and Wilson and Hemmati-Brivanlou, 1995). In *Twsg1*$^{-/-}$ mammary glands, the cytokeratin-14 positive cells within the luminal compartment may represent those cells whose BMP exposure was not sufficient to induce *Gata-3* expression preventing progression towards a terminal

luminal fate. In line with this, cells shed into the lumen of *Twsg1*$^{-/-}$ mammary glands are cytokeratin-14 positive whereas a previous study demonstrated that deleting *Gata-3* after luminal specification did not reduce cytokeratin-18 expression showing that GATA-3 is not required for maintenance of cytokeratin-18, only its induction (Kouros-Mehr et al., 2006). This suggests that *Twsg1* acts upstream of *GATA-3* to regulate epithelial/myoepithelial cell fate.

In summary, this study sheds light on the role of the extracellular regulation of BMP signaling by TWSG1 during postnatal mammary gland development. It shows that BMP signaling is reduced in mammary gland leading to a decrease in *Msx2* and *Gata-3* expression. In the absence of TWSG1, delayed ductal elongation, impaired branching and lumen formation, incomplete myoepithelial restriction, and shedding into the lumen are observed. Understanding how TWSG1 regulates normal ductal maturation can provide insight into what role it may be playing during carcinogenesis, especially in light of the recent data suggesting tumor suppressor effects of BMPs (Loh et al., 2008, Owens et al., 2011 and Ye et al., 2009). There is some evidence that the expression of Bmps, *Msx2*, and *Twsg1* is altered in breast cancer (Finak et al., 2008, Malewski et al., 2005 and Phippard et al., 1996), but the mechanisms for this differential regulation are unknown. Future studies will further elucidate the mechanism by which the lumens are eventually cleared in the absence of TWSG1. This may provide insight into how cells within the

mammary gland that escape targeted apoptosis at one point in development are identified and eventually cleared from the mammary gland.

Chapter 3: Overexpression of Twisted gastrulation, an extracellular regulator of bone morphogenetic protein signaling, promotes cell migration and invasion in breast cancer cells

Introduction

Developmental signaling pathways, such as bone morphogenetic proteins (BMPs), are often co-opted, dysregulated and/or inappropriately reactivated during the initiation, progression and invasion of cancer (Clement et al., 2000; Katsuno et al., 2008; Steinert et al., 2008). It is becoming increasingly clear that the molecular mechanisms, that maintain and promote pluripotency during development, reemerge as important mediators of tumorigenicity (Balboni et al., 2013; Buijs et al., 2012). Understanding of how this reactivation occurs (Godfrey et al., 2012; Neman et al., 2013) and what the cellular consequences of reactivation are (Ghosh et al., 2012; Maemura et al.), will provide a solid basis for advances in cancer diagnosis and future therapeutics that target these pathways (Langenfeld et al., 2013).

BMPs have been shown to be indispensable for normal embryonic development of mammary gland, lung, kidney, colon, prostate and other organs and tissues (Bellusci et al., 1996; Beppu et al., 2008; Forsman et al., 2012; Hogan, 1996; Petryk et al., 2004; Simic and Vukicevic, 2005) and are integral to basic biological processes such as cell proliferation, stem cell maintenance, migration, differentiation, apoptosis and stromal-

epithelial interactions (Lind et al., 1996; Marazzi et al., 1997; Massagué, 1998; Mukhopadhyay et al., 2006; Xia et al., 2012; Ying et al., 2003). Intriguingly, in tissues where BMPs are integral for proper development, BMPs and/or BMP pathway components are often dysregulated in cancers of that tissue (Alarmo et al., 2006; Blish et al., 2008; Langenfeld et al., 2005).

The diverse and varied set of BMP-dependent processes are only possible through precise modulation of target cell response by controlling both timing of BMP exposure and signal intensity (Arnold et al., 2006; Bonilla-Claudio et al., 2012; Obradovic Wagner et al., 2010). Establishing and maintaining the correct timing and signal intensity requires controls at multiple levels including regulating BMP in the extracellular environment. One of the major extracellular regulators of BMP signaling is Twisted gastrulation-1 (TWSG1) protein (Oelgeschlager et al., 2000; Petryk et al., 2004; Ross et al., 2001). We have previously shown that the loss of TWSG1 in the developing mammary gland leads to a reduction in BMP signaling and a delay of ductal elongation, occluded lumens and incomplete myoepithelial compartmentalization (Forsman et al., 2012) highlighting its role in normal mammary gland development. Whether TWSG1 can also regulate the behavior of breast cancer cells is currently unknown.

Several studies have identified aberrant BMP signaling in breast cancer (Alarmo and Kallioniemi, 2010; Buijs et al., 2011a; Davies et al., 2008). Additionally, expression studies have shown that *TWSG1* is also misregulated in breast cancer, most often being significantly overexpressed (Finak et al., 2008). Because TWSG1 is an important regulator

of BMP signaling, this study sought to determine if TWSG1 overexpression can directly influence breast cancer behavior and whether altered BMP signaling is involved.

As shown in transcriptional profiling studies, breast cancer is molecularly diverse, with individual tumors showing different expression profiles (Perou et al., 2000). Using these profiles, the following molecular subtypes of breast cancer have been identified: Luminal A, Luminal B, Human epithelial growth factor receptor 2 enriched (HER2+), triple negative (TN) and claudin-low. Luminal A tumors have a low proliferative index and express estrogen and progesterone receptors (ER/PR+) and are not enriched for human epidermal growth factor receptor 2 HER2 (HER2-). Luminal B tumors are ER/PR+ and HER2+ and if HER2- they have a high proliferative index. The luminal subtypes are the most differentiated of the subtypes and successful therapeutic strategies have been developed (von Minckwitz et al.). The HER2 enriched subtype is typically ER/PR- and triple negative breast cancer, often referred to as basal, is ER/PR- and HER2-. Claudin-low subtype is also a triple negative breast cancer with stem cell features. The triple negative subtype often has a poor prognosis as there are limited therapeutic options for these tumors. (For a summary of subtypes, receptor complement, prognosis and established cell lines see Table 2.) Since there is a great need to better understand the mechanisms regulating the biology of this breast cancer subtype, we focused this study on the effects of TWSG1 overexpression on triple negative breast cancer cell behavior.

Table 2: Breast cancer molecular subtypes and established cell lines.

Molecular Subtype	Receptor expression	Prognosis	Cell lines	References
Luminal A	Progesterone Estrogen	Good	MCF-7, T47D,	(Gatza et al.)
Luminal B	Progesterone Estrogen Human epidermal growth factor 2	Fair	BT474, ZR-75	(Finetti et al.)
Human epidermal receptor type	Human epidermal growth factor 2	Fair	SKBR3, MDA-MB-453	(Ades et al.)
Triple negative breast cancer		Poor	HCC-38	(Dieci et al.)
Claudin-low	Stem cell like	Poor	Bt549, MDA-MB-231, Hs578t, SUM1315	(Liu et al.)

Materials and Methods

Cell culture and reagents

Breast cancer cell lines MCF10A (non-transformed), and triple negative breast cancer cell lines HCC 38, Hs578T MDA-MB-231 and Bt549 were maintained according to American Type Culture Collection guidelines. Briefly, growth medium for MCF10A was Mammary Epithelial Basal Media (MEBM) with MEBM SingleQuot additives (Bovine Pituitary Extract 13mg/ml; hydrocortisone 0.5mg/ml; human epidermal growth factor 10mg/ml; insulin 5 mg/ml) included in the MEBM Bullet kit (Lonza/Clonetics, Allendale, NJ) and 100 ng/ml cholera toxin (Millipore, Billerica, MA). The cell line HCC-

38 was maintained in Roswell Park Memorial Institute 1640 (Sigma-Aldrich, St. Louis, MO.), 10% fetal bovine serum (FBS) (Clontech, Mountain View, CA) and 1% penicillin/streptomycin (CellGro, Manassas, VA). Bt549 used the same medium as HCC-38 with the addition of 0.01mg/ml insulin. The cell line MDA-MB-231 was grown in Dulbecco's modified epithelial medium (DMEM) (Sigma-Aldrich) with 10% FBS and 1% penicillin/streptomycin. Hs578T used the same medium as MDA-MB-231 with the addition of 0.01 mg/ml insulin. All cell lines were grown at 37°C in 5% CO2.

Biopsy Samples

Normal breast tissue shown in Figure 14B and papillary cancer shown in Figure 14G were obtained using tissue microarray (TMA) slides (a gift from Dr. Schwertfeger). Specimens and associated clinical data were obtained from the University of Minnestoa (UMN) BioNet core facility (www.bionet.umn.edu) after approval from the UMN Institutional Review Board (IRB). Coded specimens and data were provided for this study and slides containing both normal breast tissue and papillary cancer were used for immunohistochemistry. Patient identifiers were not available to the authors, but were held by the BioNet office per the BioNet IRB approval. Archival formalin-fixed paraffin-embedded tissues from patients with breast cancer treated at the UMN were collected and areas of invasive carcinoma were verified by a pathologist. The TMA core size used was 1.5 mm in diameter.

Invasive breast carcinoma samples shown in Figure 14C-F were obtained from the University of Alberta Hospital and approved by the institutional Human Research Ethics Board (University of Alberta, Edmonton, Canada and operationally by the provincial Health Care Provider (Alberta Health Services). The TMAs were made using the Beecher tissue microarrayer (model TMA-1, Estigen, Estonia) according to vendor's instructions. The TMA core size used was 1.5 mm in diameter. Archived slides stained with H&E were reviewed by two pathologists to assess tissue viability and consistency of diagnosis.

cDNA generation, RT-PCR and qRT-PCR

Total RNA was isolated from primary tumor biopsies as previously described, (Sladek et al., 2002) or cell lysates using RNeasy micro kit (Qiagen, Germantown, MD) following manufacturer's instructions. Reverse transcription was carried out with the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA) priming with an equal mix of Oligo (dT)20 and random hexamers, followed by RT and qRT-PCR (Q-PCR, MX3000p, Agilent, LaJolla, CA) for BMP pathway components. Primer sequences can be found in Table 3.

Table 3: BMP pathway components primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
BMPR1A	tagccacatcttggaggagtcg	gagtctgatttcatcccagtgcc
BMPR1B	gctcttgccgtcttgctcattc	gatagtgccaacctcgcttatgg
BMPR2	gctaaaatttggcagcaagc	cttgggccctatgtgtcact
BMP2	gtcctgagcgagttcgagtt	gcatcttgcatctgttctcg
BMP4	tgagcctttccagcaagttt	cttccccgtctcaggtatca
BMP7	tagccatttcctcaccgacg	tggagcacctgataaacgctg
MSX1	gctagaggccatgtctcctg	ccccagagcaaatgttttgt
MSX2	aatggctgcaaaacctatgc	agggagaggaaacccctttga
ID1	cgcctcaaggagctggtgcc	caggaacgcatgccgcctcg
ID2	tgcccagcatcccccagaaca	cagaagcctgcaaggacaggatg
ID3	cgctgagcttgctggacgaca	cggctgtctggatgggaaggtg
chrdl1	catcgcagttatagcgaccg	agggtgctcaaacaggacac
CHRDL1	ttcagaatcggcaacccaatc	agtgagagcggtggtaagaatgtc
TWSG1	cgacccggcggggatctagg	cagcactcgtcccaaagggcc
CYPB	tgagaccagcagatagagccaagc	tccctgccaatttgacatcttc

Immunohistochemistry

Tissue sections from normal breast shown in Figure 14B and papillary cancer shown in Figure 14G (a gift from Dr. Schwertfeger) were deparaffinized with xylenes, and rehydrated through graded alcohols (70% to 100%). Rehydrated sections were equilibrated in PBS and washed with ddH_2O, then PBS and placed in 3% H_2O_2 for 10 minutes to block endogenous peroxidases. Slides were then blocked in PBS containing 5% goat serum (Invitrogen) and 0.3% Triton X-100 (Fisher Scientific, Waltham, MA) for 60 min at room temperature. Sections were incubated with anti-TWSG1 (Abnova) 1:50 overnight at 4°C, washed and then incubated with Vectastain Elite ABC reagent (PK-

7100) and subjected to colorimetric detection with DAB substrate (Vector Laboratories, SK-4105). Tumor tissue sections shown in Figure 14C-F were incubated with a monoclonal antibody against human TWSG1 (Abnova Corporation, Taipei, Taiwan) at 1:25 dilution at 37°C for 32 min and automatically stained using a Discovery XT Ventana autostainer.

Immunofluorescence

Cells were grown on chamber slides and fixed at room temperature for 6 minutes with 4% paraformaldehyde (PFA). The slides were washed with phosphate buffered saline (PBS) and blocked in 5% Goat serum in PBS. Slides were incubated with anti-pSMAD1/5/8 (Cell Signaling Technology Inc., Danvers, MA) 1:50 washed and developed either with Alexa Fluor (Invitrogen) or DyLight (AbCam, San Francisco, CA) species-appropriate fluorophore conjugated secondary antibodies. Fluorescence was visualized on a Zeiss 710 LSM Confocal Microscope using an Argon12 laser (excitation 488 nm), a HeNe laser (excitation 543 nm) a HeNe laser (excitation 633). Images were taken under identical conditions and post-acquisition manipulations were also identical.

Adenovirus Expressing *TWSG1*

A double cassette was created using the adenovirus type 5 (Ad5) vector and the AdEasy system (Agilent) following manufacturer's instructions. Briefly, the CMV promoter-driven *TWSG1* and CMV promoter-driven *eGFP* double cassette was inserted

in place of the deleted E1 region of a common Ad5 vector. This vector was cloned into pShuttle CMV plasmid. The resultant plasmid, pShuttle CMV-*TWSG1;CMV-eGFP*, was linearized with *PmeI* digestion and subsequently co-transformed into *E. coli*. After selection of recombinants, the recombinant DNA was linearized with *PacI* digestion and transfected into transformed human embryonic retina 911 cells to generate Ad-T. The virus was propagated in HEK293 cells, dialyzed in phosphate-buffered saline with 10% glycerol, and stored at −80°C. Titering was performed with a plaque-forming assay using 911 cells (pfu/mL) and optical density-based measurement (Vp/mL). An identical replication-incompetent CMV promoter-driven *eGFP* expression vector (Ad-C) was used as a control.

Adenovirus Infection of Breast Cancer Cell lines

Cells were serum starved 24 hours prior to infection. Starved cells were treated with TrypleLE (Life Technologies, Grand Island, NY), suspended in 5ml starvation media and counted. Infection was carried out at 100pf/100cells. Cells were plated at required density for the given assay and the transduction medium was removed after 12 hours and replaced with serum free medium.

Western blotting

Cells were digested with TrypleLE (Life Technologies) with 0.5mM EDTA, incubated until cells detached, pelleted, and washed with PBS. Cells were lysed in modified mammalian protein extraction reagent (MPER) (Thermoscientific) using Halt

Protease Inhibitor (Thermoscientific) and PhosSTOP (Roche, Madison, WI). SDS-PAGE was used to separate proteins (Life Technologies) on a Nextgel 12% polyacrylamide gel (AMRESCO, Solon, OH). Proteins were transferred to PVDF Immobilon membrane (Millipore) and blocked for 1 hour in 0.1% casein buffer (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C with anti-pSMAD1/5/8 (Cell Signaling) 1:1000, or anti-Total SMAD1/5/8 (Cell Signaling) 1:1000, or anti-TWSG1 1:500, or anti-GAPDH (Cell Signaling) 1:5000 antibodies and washed 3 x 20 min with tris buffered saline and 1% Tween 20 (TBST) before incubation with HRP or fluorescent conjugated species specific antibodies. Membranes were analyzed using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) using the manufacturer's protocol. pSMAD signal intensity was normalized to Total SMAD which was first normalized to GAPDH.

Proliferation Assay

Cell proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following manufacturer's instruction. Briefly, transduced cells were plated in a 96 well plate at 1.6 x 10^5 cells/well in triplicate and serum starved for 24 hours in 100µl of serum free medium. After starvation 20µl CellTiter 96 AQueous One Solution Reagent was added to the well and incubated for 4 hours at 37°C in a 5% CO2 atmosphere. Absorbance at 490nm was recorded at 1 and 4 hours. Absorbance from a no cell control well was subtracted from all readings.

Apoptosis

Annexin V APC Apoptosis Detection Kit (eBioSciences, San Diego, CA) was used following manufacturer's instructions. Briefly, 1 x 10^6 cells were suspended in 100µl of Binding Buffer and 5µl of APC conjugated Annexin V added and incubated for 15 minutes at room temperature in the dark. Cells were washed 2X with binding buffer and resuspended in 200µl of binding buffer. Propidium iodide was added and cells analyzed by cytometry. A population of transduced control cells, either GFP:MCF10A, GFP:HCC 38 or GFP:MDA-MB-231, were treated with 50% dimethyl sulfoxide and used as a positive control. Fluorescence was measured using BD FACSCalibur and analyzed using FlowJo version 7.6.5 (Tree Star Inc., Ashland, OR). In addition to cytometry, ApoTox-Glo Triplex Assay (Promega) was used following manufacturer's instructions. Briefly, $1x10^5$ cells were plated in a 96 well plate in serum free media. Cells were grown for 24-30 hours. After 24-30 hours and 20µl Viability/Cytotoxicity Reagent was added and cells were incubated 90min at 37°C and fluorescence measured 400/505 and 485/520. Finally, 100µl of the provided Caspase-Glo 3/7 Reagent was added and cells and luminescence measured.

Migration and Invasion

BD transwell 8µm pore inserts or BD BioCoat Matrigel Invasion Chambers (BD Biosciences) were used according to the manufacturer's instructions. Transduced cells (5×10^4) were treated with the BMP specific inhibitor, dorsomorphin, (Sigma-Aldrich) or

vehicle in serum free media for 12 hours. Cells were seeded on top of cell inserts (migration) or rehydrated Matrigel inserts (invasion). As a chemoattractant, MEBM or DMEM containing 2 % FBS was used. Cells were incubated for 18 h at 37°C. After 18 hours the noninvading cells were removed and GFP+ cells counted in 10 random fields per well.

Zymography

Cells were lysed directly in the well with 500µl MPER (Thermoscientific) in the absence of reducing agent. After collection, 60µg of total protein were loaded onto Tris-Glycine gelatin gels and separated by electrophoresis. Gels were renatured for 60 minutes in Renaturing Buffer (Invitrogen) at room temperature. The gels were then placed in 100ml of Developing Buffer (Invitrogen) and allowed to develop for 16 hours at 37°C. Gels were then stained with Simply Blue (Invitrogen) and destained with ddH2O. Gels were digitized using a Cannon flatbed scanner.

Statistical Analysis

pSMAD signal intensity was normalized to total SMAD which was first normalized to GAPDH. Significance was calculated using a Student's t-test with significance set at $p<0.05$.

Results

Twisted gastrulation is expressed in breast carcinomas and normal breast tissue.

Published expression studies suggest that *TWSG1* is overexpressed in many primary tumors and cell lines (Finak et al., 2008). To confirm this, primary tumor cDNA was queried for *TWSG1* expression. In many primary tumors, *TWSG1* transcript was elevated, 1.0 to 3.0 fold, when compared to normal breast with no correlation of expression level to ER/PR status (Figure 14A). Additional information regarding molecular subtypes was either not collected or not known for these women.

As in mouse, TWSG1 is restricted to the myoepithelium in the normal breast as shown by immunohistochemistry (Figure 14B). However, it is widely expressed in invasive ductal carcinoma (Figure 14C), invasive lobular carcinoma (Figure 14D), invasive papillary carcinoma (Figure 14E & G) and ductal carcinoma in situ (Figure 14F). In invasive carcinoma, TWSG1 does not remain restricted to the myoepithelium.

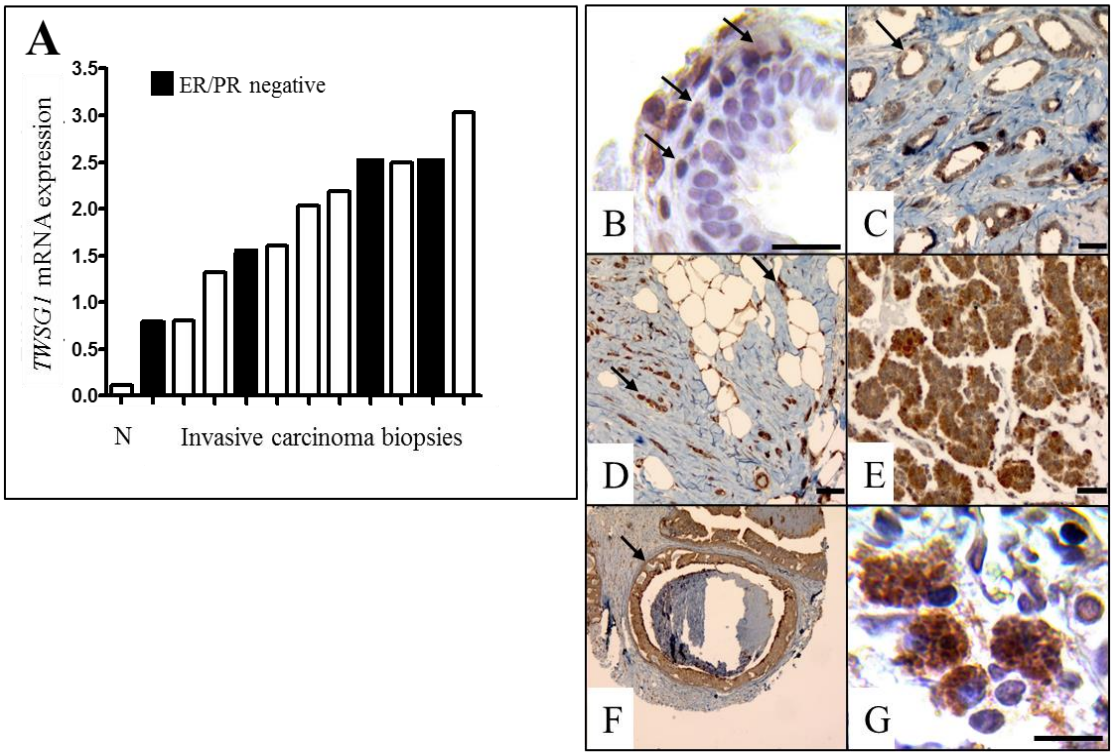

Figure 14: TWSG1 is expressed in breast cancer tissue.
A. cDNA libraries were generated from breast tumors that were collected from women diagnosed with invasive carcinoma. These libraries were then queried for *TWSG1* expression. *TWSG1* expression was elevated in a subset invasive cancers when compared to normal tissue. Normal tissue (N) was obtained from a breast biopsy and expression levels were normalized to RPLPO. TWSG1 was detected by immunohistochemistry in **B.** normal breast tissues (400X), arrows show myoepithelial expression of TWSG1 **C**. invasive ductal carcinoma **D**. invasive lobular carcinoma **E.** invasive papillary carcinoma (C, D and E 200X, scale bar= 10 micrometers, B and G 400X) **F.** ductal carcinoma in situ (50X) **G**. papillary carcinoma.

Representative cell lines from each molecular subtype were assayed for *TWSG1* expression and we found that some triple negative breast cancer cell lines, including MDA-MB-231 and Bt549, expressed significantly more *TWSG1* than non-transformed cells, suggesting that T*WSG1* may be increased in a subset of triple negative breast cancers. Due to the difficulty of treatment and poor prognosis of these types of tumors, the remainder of our study focused on this molecular subtype. Of the triple negative breast cancer cell lines

that were assayed (Figure 15A), we selected a low *TWSG1* expression line, HCC 38, and a high *TWSG1* expressing cell line, MDA-MB-231, for our functional studies.

BMP pathway components are variably expressed in triple negative breast cancer cell lines

To understand which components of the BMP pathway are present in normal and breast cancer cells, RTPCR was used to evaluate the BMP pathway transcriptome in normal and selected triple negative breast cancer cell lines: HCC 38, Hs578T, MDA-MB-231, and Bt549 (Figure 15B). MCF10A cells were used as a non-transformed control. The BMP ligands and extracellular partners were differentially expressed between control and triple negative breast cancer cell lines. For example, the extracellular BMP antagonist *CHORDIN* was only detectable by RTPCR in non-transformed controls, but not breast cancer cells. *TWSG1* high expressing cell lines expressed another Chordin family member, *CHORDIN-LIKE 1*. Additionally, most of the triple negative breast cancer cell lines queried preferentially expressed BMP 4, but not BMP 2 except for Bt549 (Figure 15B,C). This is in line with previously published studies (Alarmo et al., 2007; Rodriguez-Martinez et al., 2011) We also assayed for well-defined BMP target genes (*MSX1, MSX2, ID1, ID2* and *ID3)* and found no clear difference between tumor cell lines and non-transformed control (Figure 15B).

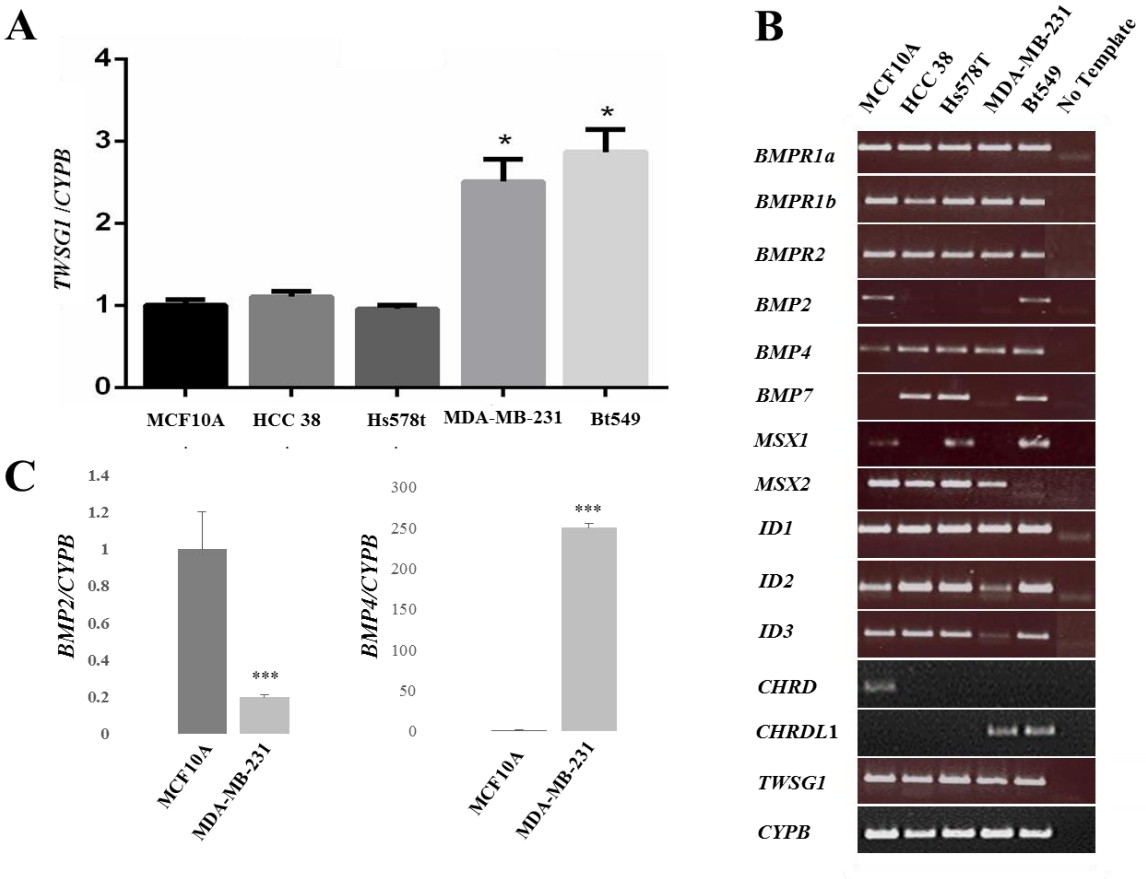

Figure 15. In some triple negative breast cancer cells *TWSG1* expression is elevated. A. qRTPCR comparing *TWSG1* transcript in MCF10A control cells and triple negative cell lines. (* = P< 0.05) **B.** RTPCR showing the BMP pathway transcriptome. **C.** qRTPCR showing that BMP 2 is expressed in MCF10A but not MDA-MB-231 and BMP4 is expressed in MDA-MB-231 but not MCF10A.

Overexpression of TWSG1 positively regulates pSMAD1/5/8

BMPs signal by phosphorylating their intracellular signal transducer, pSMAD 1/5/8. To determine what effect overexpression of TWSG1 had on BMP signaling, cells were interrogated for pSMAD 1/5/8. First, adenovirus mediated overexpression of TWSG1 was confirmed by western blot and qRT-PCR (Figure 16A,B). Non-transformed

controls (MCF10A), *TWSG1* low expressing cells (HCC 38) and *TWSG1* high expressing cells (MDA-MB-231) had significant increases in pSMAD 1/5/8 when normalized to the total SMAD 1/5/8 pool, indicating an increase in BMP signaling. To determine cellular localization, cells were stained for pSMAD 1/5/8 and DAPI. We found an increase in nuclear localization of pSMAD 1/5/8, as observed by signal intensity, in cells overexpressing TWSG1 for both the control and triple negative breast cancer (Figure 17). Some GFP control cells also had pSMAD 1/5/8 localized to nucleus. However, the signal intensity and/or the number of pSMAD 1/5/8 positive cells was noticeable between GFP controls and TWSG1 overexpressing cells.

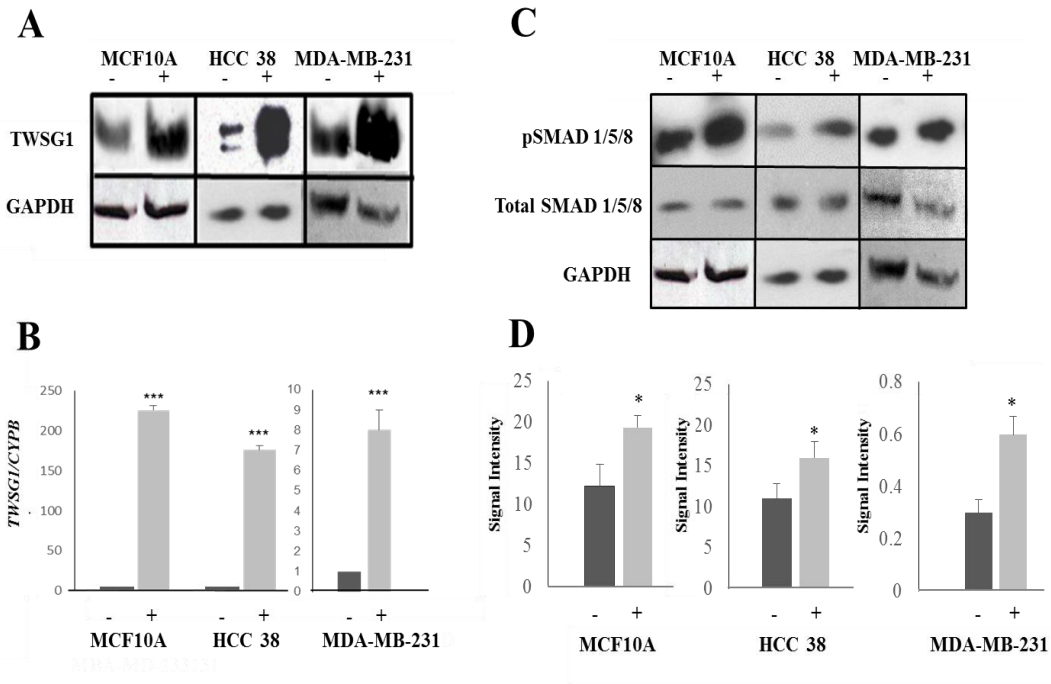

Figure 16. Transduction with CMV-eGFP,CMV-*TWSG1* (+) leads to a significant increase in *TWSG1* transcript and protein as well as pSMAD 1/5/8. **A.** There was more TWSG1 in cells transduced with CMV-eGFP,CMV-*TWSG1* (+) when compared to cells transduced with CMV-*eGFP* (-) **B**. quantification of *TWSG1* transcript by qRTPCR ($p < 0.01$) **C.** an immunoblot showing an increase pSMAD 1/5/8 in those cells overexpressing TWSG1. Total SMAD 1/5/8 and GAPDH were used as normalizers **D.** pSMAD 1/5/8 signal intensity is significantly increased in cells overexpressing TWSG1 (GAPDH/Total SMAD 1/5/8)(pSMAD 1/5/8) $p < 0.05$.

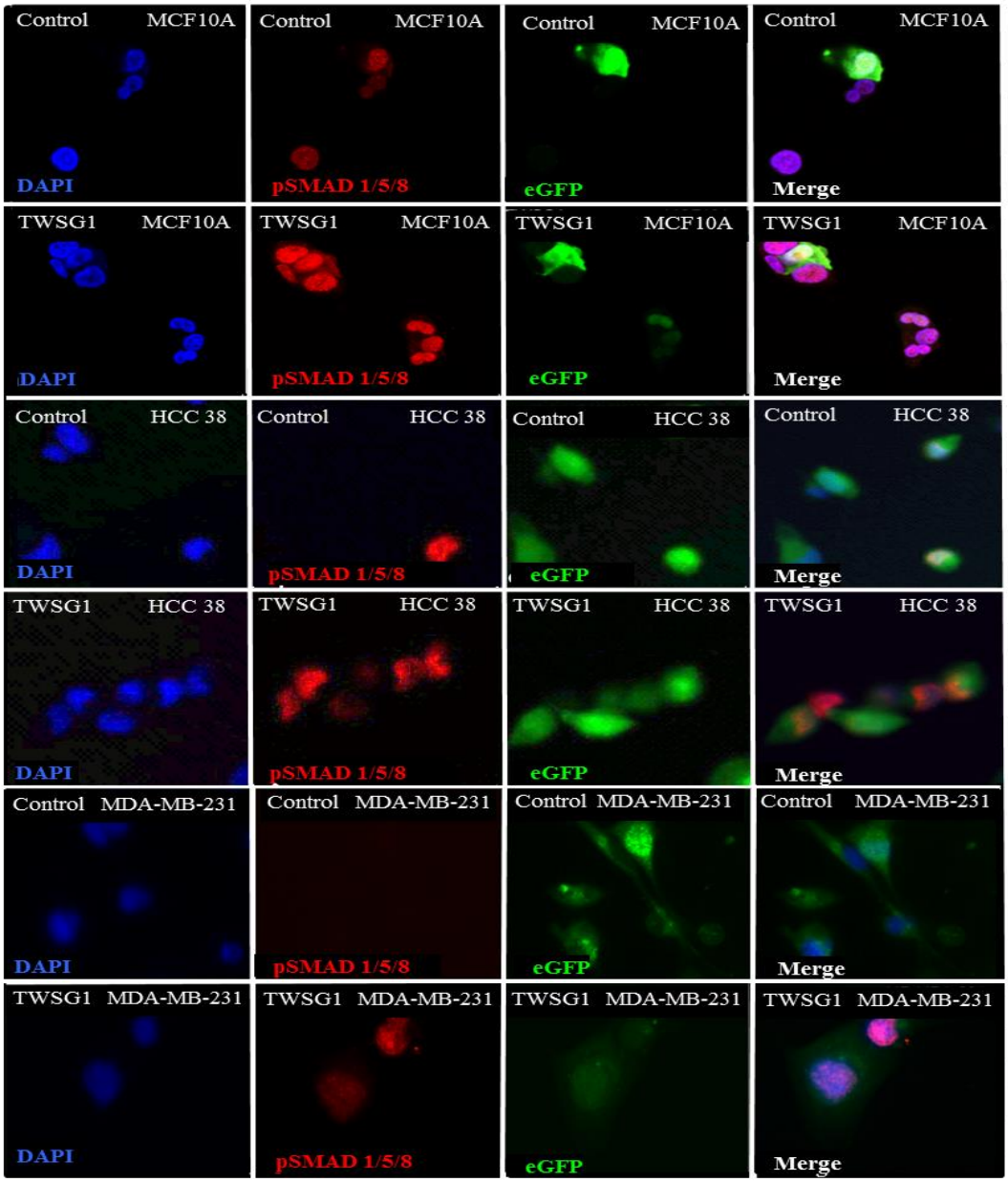

Figure 17. pSMAD 1/5/8 localization. Cells were tranduced with either CMV-*eGFP* or CMV-*eGFP*, CMV-*TWSG1*. **A.** There was an increase in pSMAD 1/5/8 localized to the nucleus in *TWSG1* overexpressing MCF10A cells **B.** pSMAD 1/5/8 localized to the nucleus in *TWSG1* overexpressing HCC 38 cells **C.** *TWSG1* overexpressing MDA-MB-231 cells also saw an increase in pSMAD 1/5/8 localized to the nucleus.

Apoptosis is increased but proliferation remains unchanged in cells overexpressing TWSG1

BMPs regulate numerous biological processes including proliferation. In cancer, proliferation is a prerequisite for tumor formation. To investigate if increased BMP signaling led to an increase in proliferation, a dimethyl thiazolyl diphenyl tetrazolium salt (MTT) assay was completed. There was no significant difference in proliferation in any of the cell lines measured (Figure 18A). BMP signaling also regulates apoptosis and we found that overexpression of *TWSG1* in non-transformed controls and in HCC 38 led to a significant increase in apoptosis in those cell lines (Figure 17B). However, there was no significant difference in apoptosis in *TWSG1* overexpressing MDA-MB-231 cells (Figure 17B), possibly due to the already high levels of TWSG1 present in these cells.

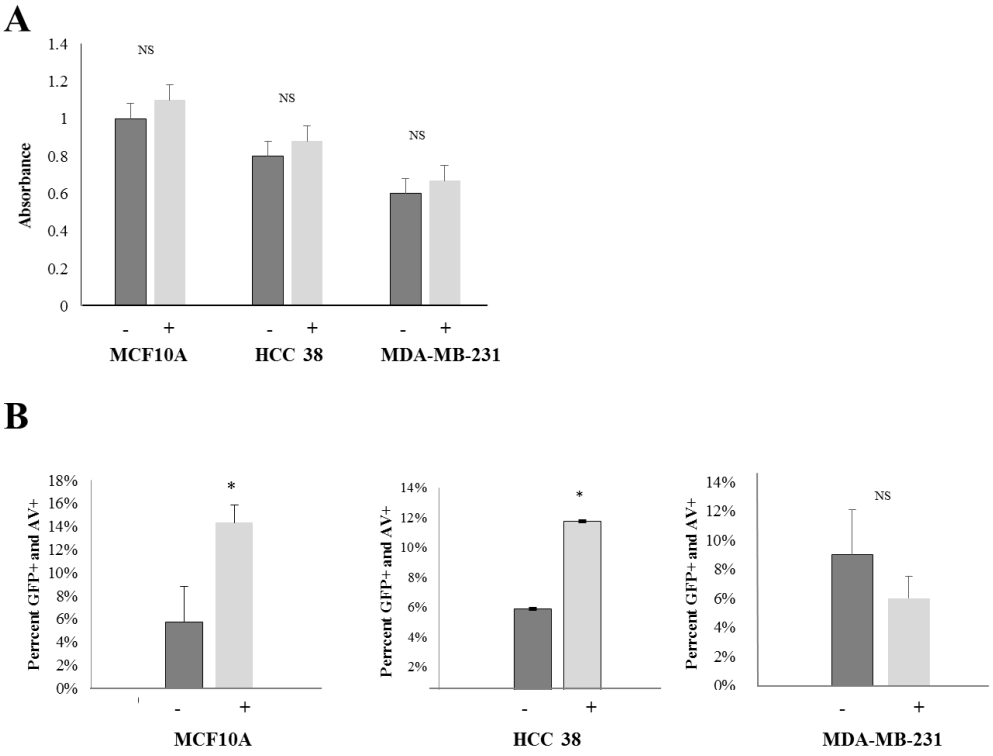

Figure 18. Apoptosis was increased in both the non-transformed control and low *TWSG1* expressing tumor cells (HCC 38). Proliferation was unchanged A. MTT assay showed no difference in proliferation between control and *TWSG1* overexpressing cells **B.** Cytometric analysis of cells that were both GFP+ and Annexin V+ indicating those cells in early apoptosis. * $p<0.05$

TWSG1 promotes migration and invasion in a BMP dependent manner

BMPs play a significant role in cell migration during normal development (Christiaen et al., 2010) and can induce the expression of early epithelial mesenchymal transition (EMT) genes, an early step in invasion. (Gonzalez and Medici, 2014). To examine the migratory and invasive potential of breast cancer cells over expressing TWSG1, serum starved *TWSG1* overexpressing cells were plated into transwell inserts with and without matrigel.. *TWSG1* overexpressing cells migrated significantly better than control transduced cells (Figure 19A). Additionally, all cell lines overexpressing *TWSG1*

invaded significantly better than control (Figure 19B). The increases in migration and invasion is a BMP-dependent process as cells that were treated with the BMP specific inhibitor, dorsomorphin, (Figure 19B) migrated and invaded no better than GFP expressing controls when. Significance was determined by comparing control to *TWSG1* overexpressing cells, then comparing *TWSG1* overexpressing cells to dorsomorphin treated cells. Finally, control cells were compared to dorsomorphin treated *TWSG1* overexpressing cells using a Student's t-test.

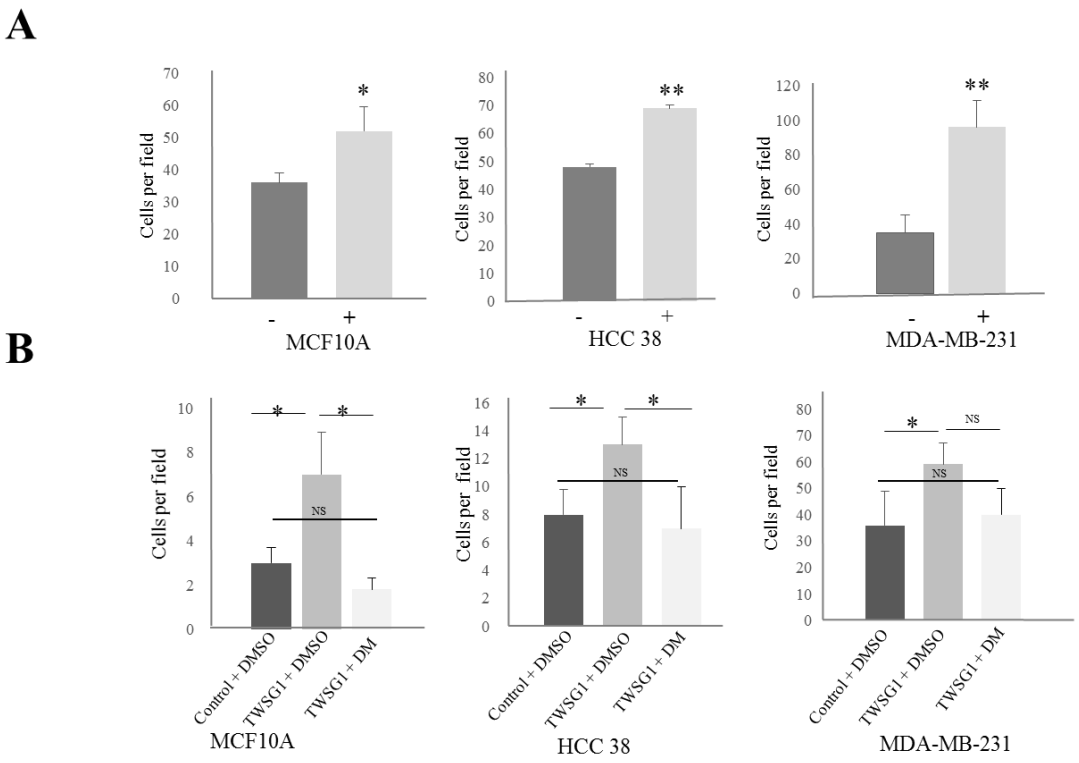

Figure 19. Invasion and Migration are increased as a result of TWSG1 overexpression. A. Transwell migration assay shows increased migration in TWSG1 overexpressing cells. **B.** Transwell invasion assays show an increase in migration in TWSG1 overexpressing cells. When cells are treated with dorsomorphin, invasion was inhibited. *p<0.05, **p<0.01

TWSG1 regulates MMP expression

In order for invasion to occur, cells must breakdown the extracellular matrix (ECM) and they accomplish this by secreting matrix metalloproteinases (MMP). It has been shown that BMPs can induce MMP expression (Ampuja et al., 2013) thereby facilitating invasion. Given the significant increase in invasion observed in *TWSG1* overexpressing cells, and the requirement for MMP activity for invasion, we assayed expression and activity of selected MMPs. One of the MMPs important in cancer metastasis is MMP 3 and it is activated early in invasion. MMP 9 and MMP 2 are collagenases that break down ECM during invasion. Interestingly, MMP 3 expression was undetectable in MCF10A *GFP* transduced cells and barely detectable in HCC 38 GFP transduced cells but was present in MCF10A and HCC 38 cells overexpressing *TWSG1* (Figure 20A.) We also investigated MMP activity by zymography and found both MMP 2 and MMP 9 were more active in all *TWSG1* transduced cells (Figure 20B) except for MMP 9 in MDA-MB-231 cells. This is not surprising as this cell line is reported as being metastatic.

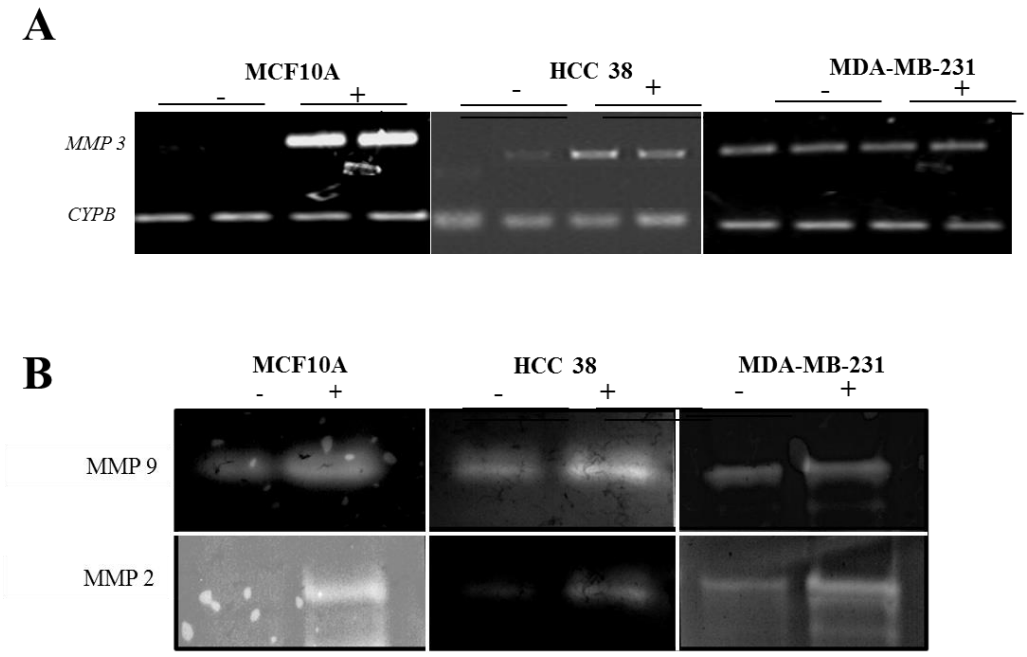

Figure 20. **MMP 3 expression is increased in TWSG1 overexpressing cells A.**RTPCR shows absence and/or presence of MMP 3 in MCF10A and HCC 38 cells but not MDA-MB-231 cells. **B.** Zymography showing MMP 9 and MMP 2 activity increases in TWSG1 overexpressing cells except for MMP 9 in MDA-MB-231 cells.

Discussion

BMPs are developmentally important molecules that regulate numerous processes during embryogenesis such as proliferation, differentiation and apoptosis. Current studies show that the BMP signaling pathway is reawakened during tumor initiation and progression (Rodriguez-Martinez et al., 2011; Ye et al., 2009) and mutations in the components of the BMP pathway, such as BMP receptor type IA and SMAD4, have been implicated in juvenile polyposis syndrome (Montesano, 2007) but are rare in cancer. It is not uncommon for BMPs to be reported as both tumor promoting and tumor suppressing (Owens et al., 2011; Shon et al., 2009). It is thought that early on BMPs may play a

suppressive role by limiting proliferation and in later disease become tumor promoting by facilitating invasion. Ultimately, BMP signaling outcomes are dependent on which ligands are being expressed and which extracellular BMP binding partners are also being expressed such as TWSG1.

In the present study, we show that TWSG1 is overexpressed in invasive breast cancer including ductal, lobular, and papillary carcinoma. Furthermore, expression of TWSG1 is no longer restricted to the myoepithelium as it is in the normal breast. Using adenovirus mediated *TWSG1* overexpression in selected triple negative breast cancer cell lines we show that *TWSG1* is acting, as it does during normal development in the mammary gland, to positively regulate BMP signaling via pSMAD 1/5/8 (Forsman et al., 2012). In cancer, there is a requirement for uncontrolled proliferation and cell survival BMP regulated processes. We determined that while proliferation was unaffected, the apoptotic rate changed significantly upon overexpression of TWSG1. Cells that basally express low levels of *TWSG1,* HCC 38 and MCF10A, the apoptotic rate was significantly increased when TWSG1 was overexpressed. Conversely, the apoptotic rate in triple negative breast cancer cells that basally express high levels of *TWSG1* (MDA-MB-231) remained unchanged when TWSG1 was overexpressed. It may be that in cells that already have a high level of TWSG1, the net increase in TWSG1 mediated BMP signaling is insufficient to trigger apoptosis while cells that express low levels of TWSG1 experience a larger net increase in BMP signaling thus triggering a different cellular response (Nishinakamura and Sakaguchi, 2014).

Our study also demonstrates that TWSG1 mediated BMP signaling increases the invasive behavior of triple negative breast cancer cells. Interestingly, we show that blocking BMP signaling returns invasive behavior of *TWSG1* overexpressing cells to that of controls. It is possible that other pathways also contribute to the invasive phenotype as invasion was not completely abolished by BMP inhibition. However, we did observe that MMP 3 expression and MMP 2 and MMP 9 activity were all enhanced in TWSG1 overexpressing cells with one exception. MMPs are required for ECM degradation allowing tumor cells to migrate out from the primary tumor. Tumor cells that overexpress *TWSG1* may be more malignant via increased BMP signaling leading to an increase in MMP expression and activation.

This study elucidates a role for TWSG1 in promoting BMP signaling leading to an increase in migration and invasion. Curiously, while there was a significant increase in pSMAD 1/5/8 levels and pSMAD 1/5/8 was localized to the nucleus well-defined BMP target genes did not appear to be changed. However, an increase in MMP activity was demonstrated. Future studies will focus on the extracellular BMP binding proteins and the different ligand interactions. We need to understand the functional consequences of expressing a particular ligand or a particular extracellular BMP binding partner.

Finally, changes in the BMP transcriptome may be useful in predicting aggressiveness and/or disease progression. The utility of TWSG1 immunoreactivity as a marker of invasive breast carcinoma also deserves further study.

Chapter 4: Conclusion and Future Directions

Development and Cancer

Studies over the last decade have shown that key developmental pathways such as BMP, wingless-related integration, transforming growth factor β, fibroblast growth factor, retinoic acid, notch and sonic hedgehog are reactivated during tumorigenesis (Bailey et al., 2007; Bocchinfuso et al., 1999; Lewis and Visbal, 2006; Piek et al., 1999). This is not unexpected as tumor cells exhibit many of the same behaviors as embryonic cells such as proliferation, migration, invasion, colonization of distant sites and modification of the microenvironment to support survival and growth (Friedl and Gilmour, 2009; Howard and Lu, 2014; Kaufhold and Bonavida, 2014). Cancer, viewed through the lens of development, looks remarkably like dysregulated morphogenesis.

In this study we highlight a link between mammary gland development and breast cancer. First, we identified a role for TWSG1 in mammary gland development through its ability to modulate BMP signaling. TWSG1's positive regulation of BMP signaling is required for complete luminal cell fate determination through GATA-3. Extending our findings, we hypothesized that TWSG1 may also have a role in breast cancer. Overexpression of TWSG1 in triple negative breast cancer cells led to an increase in invasive behavior and upregulation of MMP expression and activity. In the developing mammary gland, further analysis will focus on clarifying the molecular players that lead to the loss of myoepithelial compartmentalization, occluded lumens and delayed

elongation. In breast cancer, future studies will investigate TWSG1 mediated BMP signaling in tumor formation and invasion.

In *Twsg1*$^{-/-}$ mammary glands, we observed reduced BMP signaling and subsequent reduction in GATA-3 expression. Quite striking was the observation of GATA-3 negative cells residing in the luminal compartment. GATA-3 is a transcription factor that is necessary and sufficient for the differentiation of bipotent progenitors into mammary luminal cells (Figure 21) (Kouros-Mehr et al., 2008). Our next step will need to determine if GATA-3 negative cells disrupt cell:cell contacts in the luminal compartment. This disruption, if it exists, may account for the delay in elongation as mechanical force generated by luminal cells contribute to elongation (Shebanova and Hammer, 2014). To test this theory, GATA-3 could be knocked down in luminal cells and the rate of elongation monitored.

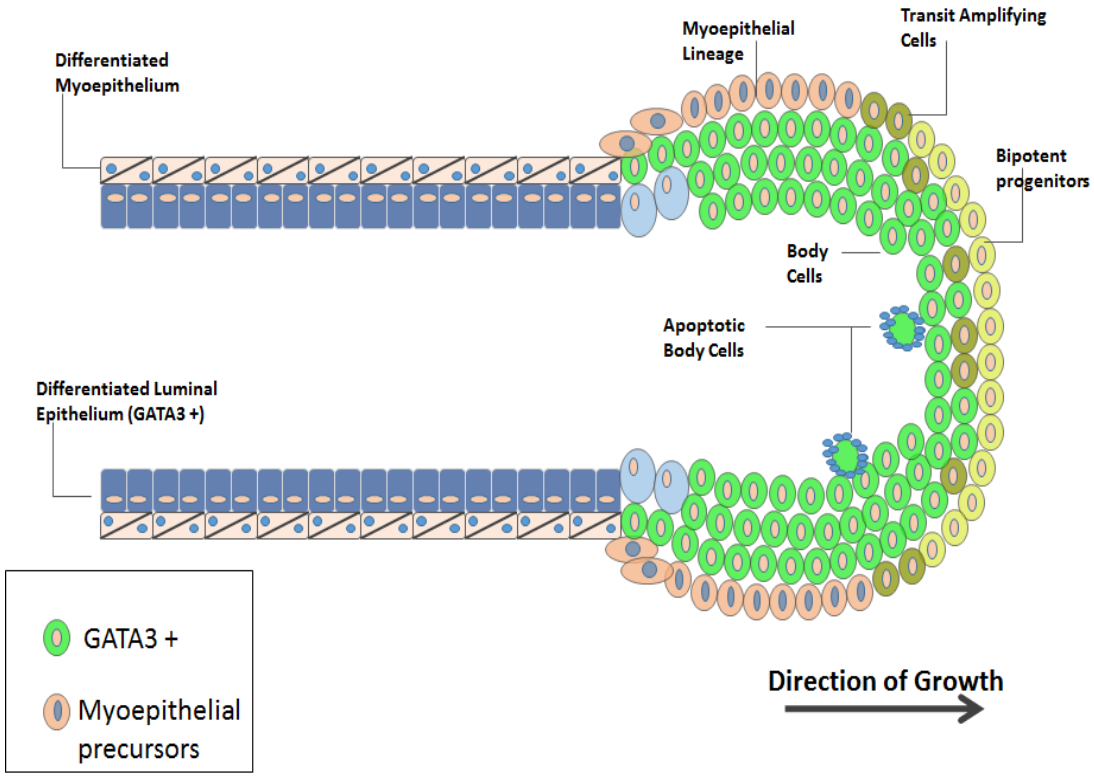

Figure 21: Schematic of terminal end bud. The terminal end bud is that site of extensive proliferation and apoptosis in the elongating duct. Bipotent progenitors give rise to GATA3 negative myoepitheal cells and GATA3 positive luminal cells.

In our previous study, we were unable to determine if GATA-3 negative cells within the luminal compartment had once expressed GATA-3 but lost expression in the low BMP environment or if that particular cell never received sufficient BMP signaling to express GATA-3. The latter suggests that BMP is upstream of GATA-3 and is required for luminal specification. The former would suggest that BMPs are needed to maintain luminal identity. Future studies will repress GATA-3 in cells that are resident in the luminal compartment, treat those cells with BMP to see if GATA-3 can be restored. In cancer, GATA-3 expression is positively correlated with relapse free survival and a good

prognosis. Reestablishing luminal identity in cancer cells may provide additional therapeutic avenues.

In the normal mammary gland, TWSG1 expression is restricted to the myoepithelium but observed pSMAD 1/5/8 nuclear localization was seen in the luminal compartment (Figure 9B) suggesting paracrine signaling. It would be interesting to construct a membrane bound version of TWSG1, express it in the myoepithelium and determine if pSMAD 1/5/8 continues to be present in the luminal compartment. We suspect that the ability of TWSG1 to bind BMP and diffuse is critical for luminal cell fate determination and that a membrane bound TWSG1 would be unable to diffuse and therefore luminal specification would be dysregulated.

Interestingly, we did not see an expansion of the myoepithelial layer rather GATA-3 negative cells were found within the luminal compartment or being shed into the lumen. Simply put, it has been shown that developmental programs can be used to induce pluripotent cells from terminally differentiated cells (Wang et al., 2011a). Therefore, it may be possible to use developmental programs to drive tumor cells back towards terminal differentiation and perhaps make them vulnerable to immune surveillance or targeted therapeutics.

Apoptosis was delayed but ducts were eventually cleared and lumens formed. Future studies will focus on the mechanism(s) by which the lumens are eventually cleared in the absence of TWSG1. This may provide insight into how cells within the

mammary gland that escape targeted apoptosis at one point in development are identified and eventually cleared.

In human breast cancer very few mutations, amplification and/or deletions were detected in the BMP pathways (Peltoketo et al., 2004). However, BMP pathway dysregulation occurs in a large percentage of breast cancer despite lack of direct gene mutations. (Kuhl and Kuhl, 2012). .

Developmental studies can help refine our understanding of tissue specific molecular action. For example, our lab has previously shown that TWSG1 acts primarily as an antagonist in the developing face (Billington et al.; MacKenzie et al., 2009) but as an agonist in the developing mammary gland (Forsman et al., 2012). When we overexpressed *TWSG1* in triple negative breast cancer cells we saw that it remained an agonist and pSMAD1/5/8 was significantly increased (Figure 16C-D). To test the idea that *TWSG1* retains its developmental function, agonist or antagonist, we assessed oral squamous cell carcinoma. Lesions were stained for TWSG1by immunohistochemistry and we observed that TWSG1was expressed in the proliferative zone and in migrating epithelial rafts (Figure 22). When the lesions were interrogated for pSMAD1/5/8 we found expression dramatically reduced in the proliferative zone in SCC. This finding seems to be consistent with the role TWSG1plays during craniofacial development.

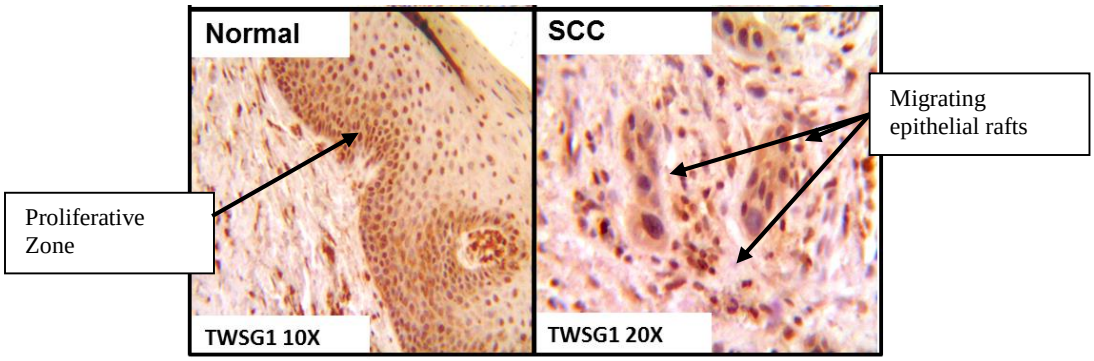

Figure 22: TWSG1is expressed in normal oral tissue as well as SCC. TWSG1is present in the normal oral epithelium at the site of proliferation. In squamous cell carcinoma (SCC), TWSG1was observed in the migrating epithelial rafts.

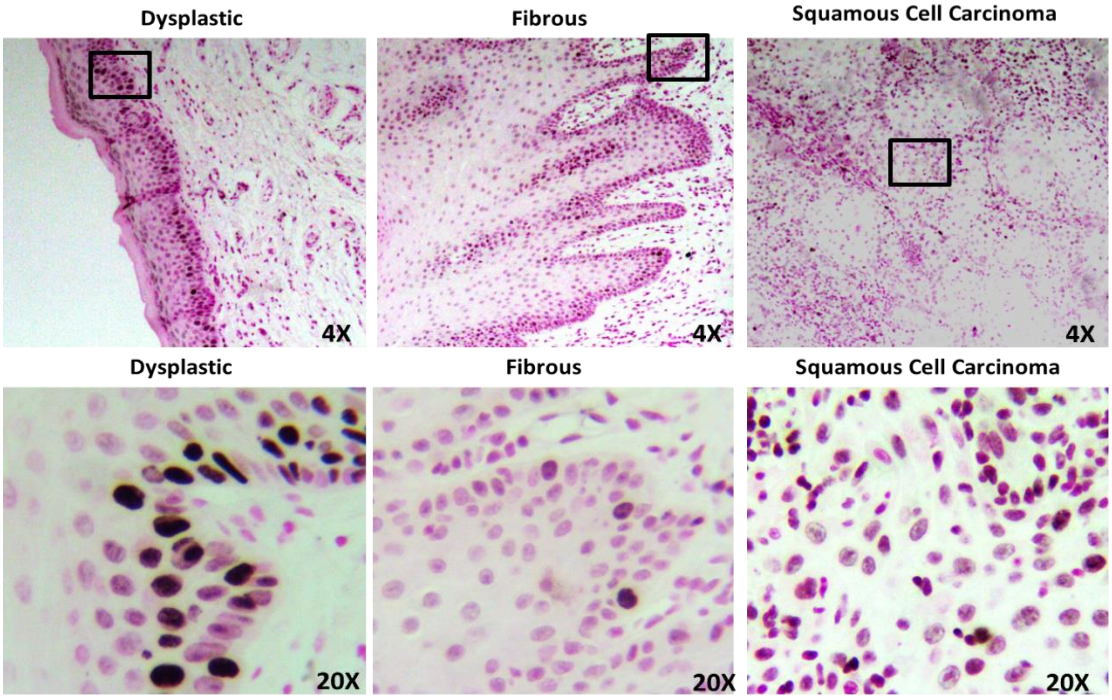

Figure 23: pSMAD is reduced in SCC. In the proliferative zone of normal oral epithelium many cells were positive for nuclear pSMAD. That signal seems to be diminished in SCC.

Future Directions

Using breasts cancer cells future studies will focus on the functional consequences of expressing different BMP ligands, BMP binding proteins and their interactions in regulating aggressiveness and/or disease progression. We know that ligand composition can influence the cellular response, we also know that preformed complexes can change the signaling intensity and thus the cellular outcome. Defining the microenvironment in terms of BMP and BMP binding proteins will provide insight into the different role TWSG1 plays in breast cancer when compared to other tissues. One can imagine that the extracellular partners may be different. Once the partners are identified, it would be intriguing to create a breast tumor niche and observe how squamous cells respond and conversely how BCCs respond in an oral niche. If changes in pSMAD1/5/8 occur are there also changes in migratory and invasive behavior.

Conclusion

Our considerable understanding of normal development and the molecular mechanisms that govern these numerous and integrated processes, will allow us to better understand how these mechanisms are circumvented and/or coopted during tumorigenesis. Like some cells during development, tumor cells have the ability to proliferate, go through epithelial to mesenchymal transition, migrate through the body and influence distant tissues to provide a suitable environment for colonization and growth by, as one author quipped, “tumoral abuse of the developmental [pathway]” (Sailer et al., 2013). Leveraging our

understanding of development could provide novel and effective cancer treatments thereby improving the outcome for those afflicted with this disease.

References:

Chapter 2 note of previous publication

Figures, tables and text in Chapter 2 are reprinted with minor editing from the article:

Forsman, C. L., Ng, B. C., Heinze, R. K., Kuo, C., Sergi, C., Gopalakrishnan, R., Yee, D., Graf, D., Schwertfeger, K. L. and Petryk, A. (2012). BMP-binding protein twisted gastrulation is required in mammary gland epithelium for normal ductal elongation and myoepithelial compartmentalization. *Dev Biol* **373**, 95-106.

Journal Authors retain "the right to include the journal article, in full or in part, in a thesis or dissertation" (http://www.elsevier.com/wps/find/authorsview.authors/rights#whatrights)

Works Cited

Ades, F., Zardavas, D., Bozovic-Spasojevic, I., Pugliano, L., Fumagalli, D., de Azambuja, E., Viale, G., Sotiriou, C. and Piccart, M. Luminal B Breast Cancer: Molecular Characterization, Clinical Management, and Future Perspectives. *Journal of Clinical Oncology* **32**, 2794-2803.

Alarmo, E. L. and Kallioniemi, A. (2010). Bone morphogenetic proteins in breast cancer: dual role in tumourigenesis? *Endocr Relat Cancer* **17**, R123-39.

Alarmo, E. L., Kuukasjarvi, T., Karhu, R. and Kallioniemi, A. (2007). A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. *Breast Cancer Res Treat* **103**, 239-46.

Alarmo, E. L., Rauta, J., Kauraniemi, P., Karhu, R., Kuukasjarvi, T. and Kallioniemi, A. (2006). Bone morphogenetic protein 7 is widely overexpressed in primary breast cancer. *Genes Chromosomes Cancer* **45**, 411-9.

Ampuja, M., Jokimaki, R., Juuti-Uusitalo, K., Rodriguez-Martinez, A., Alarmo, E. L. and Kallioniemi, A. (2013). BMP4 inhibits the proliferation of breast cancer cells and induces an MMP-dependent migratory phenotype in MDA-MB-231 cells in 3D environment. *BMC Cancer* **13**, 429.

Arnold, S. J., Maretto, S., Islam, A., Bikoff, E. K. and Robertson, E. J. (2006). Dose-dependent Smad1, Smad5 and Smad8 signaling in the early mouse embryo. *Dev Biol* **296**, 104-18.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland, R. M., Rossant, J. et al. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-661.

Bailey, J. M., Singh, P. K. and Hollingsworth, M. A. (2007). Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J Cell Biochem* **102**, 829-39.

Balboni, A. L., Hutchinson, J. A., DeCastro, A. J., Cherukuri, P., Liby, K., Sporn, M. B., Schwartz, G. N., Wells, W. A., Sempere, L. F., Yu, P. B. et al. (2013). DeltaNp63alpha-mediated activation of bone morphogenetic protein signaling governs stem cell activity and plasticity in normal and malignant mammary epithelial cells. *Cancer Res* **73**, 1020-30.

Bellusci, S., Henderson, R., Winnier, G., Oikawa, T. and Hogan, B. L. (1996). Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* **122**, 1693-702.

Beppu, H., Mwizerwa, O. N., Beppu, Y., Dattwyler, M. P., Lauwers, G. Y., Bloch, K. D. and Goldstein, A. M. (2008). Stromal inactivation of BMPRII leads to colorectal epithelial overgrowth and polyp formation. *Oncogene* **27**, 1063-70.

Billington, C. J., Jr., Fiebig, J. E., Forsman, C. L., Pham, L., Burbach, N., Sun, M., Jaskoll, T., Mansky, K., Gopalakrishnan, R., O'Connor, M. B. et al. Glycosylation of Twisted Gastrulation is Required for BMP Binding and Activity during Craniofacial Development. *Front Physiol* **2**, 59.

Blish, K. R., Wang, W., Willingham, M. C., Du, W., Birse, C. E., Krishnan, S. R., Brown, J. C., Hawkins, G. A., Garvin, A. J., D'Agostino, R. B., Jr. et al. (2008). A human bone morphogenetic protein antagonist is down-regulated in renal cancer. *Mol Biol Cell* **19**, 457-64.

Bocchinfuso, W. P., Hively, W. P., Couse, J. F., Varmus, H. E. and Korach, K. S. (1999). A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. *Cancer Res* **59**, 1869-76.

Bonilla-Claudio, M., Wang, J., Bai, Y., Klysik, E., Selever, J. and Martin, J. F. (2012). Bmp signaling regulates a dose-dependent transcriptional program to control facial skeletal development. *Development* **139**, 709-719.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and Robertis, E. M. D. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Bowman, A. and Nusse, R. (2011). Location, Location, Location: FoxM1 Mediates Î²-Catenin Nuclear Translocation and Promotes Glioma Tumorigenesis. *Cancer Cell* **20**, 415-416.

Brunet, L. J., McMahon, J. A., McMahon, A. P. and Harland, R. M. (1998). Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton. *Science* **280**, 1455-1457.

Brunkow, M. E., Gardner, J. C., Van Ness, J., Paeper, B. W., Kovacevich, B. R., Proll, S., Skonier, J. E., Zhao, L., Sabo, P. J., Fu, Y.-H. et al. (2001). Bone Dysplasia Sclerosteosis Results from Loss of the SOST Gene Product, a Novel Cystine Knotâ€“Containing Protein. *The American Journal of Human Genetics* **68**, 577-589.

Buijs, J. T., van der Horst, G., van den Hoogen, C., Cheung, H., de Rooij, B., Kroon, J., Petersen, M., van Overveld, P. G., Pelger, R. C. and van der Pluijm, G. (2011a). The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. *Oncogene* **31**, 2164-74.
Buijs, J. T., van der Horst, G., van den Hoogen, C., Cheung, H., de Rooij, B., Kroon, J., Petersen, M., van Overveld, P. G., Pelger, R. C. and van der Pluijm, G. (2012). The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. *Oncogene* **31**, 2164-74.
Buijs, J. T., van der Horst, G., van den Hoogen, C., Cheung, H., de Rooij, B., Kroon, J., Petersen, M., van Overveld, P. G. M., Pelger, R. C. M. and van der Pluijm, G. (2011b). The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. *Oncogene*.
Chen, A., Wang, D., Liu, X., He, S., Yu, Z. and Wang, J. (2012). Inhibitory effect of BMP-2 on the proliferation of breast cancer cells. *Mol Med Rep* **6**, 615-20.
Cho, K. W., Kim, J. Y., Song, S. J., Farrell, E., Eblaghie, M. C., Kim, H. J., Tickle, C. and Jung, H. S. (2006). Molecular interactions between Tbx3 and Bmp4 and a model for dorsoventral positioning of mammary gland development. *Proc Natl Acad Sci U S A* **103**, 16788-93.
Christiaen, L., Stolfi, A. and Levine, M. (2010). BMP signaling coordinates gene expression and cell migration during precardiac mesoderm development. *Developmental Biology* **340**, 179-187.
Clement, J. H., Marr, N., Meissner, A., Schwalbe, M., Sebald, W., Kliche, K. O., Hoffken, K. and Wolfl, S. (2000). Bone morphogenetic protein 2 (BMP-2) induces sequential changes of Id gene expression in the breast cancer cell line MCF-7. *J Cancer Res Clin Oncol* **126**, 271-9.
Davies, S. R., Watkins, G., Douglas-Jones, A., Mansel, R. E. and Jiang, W. G. (2008). Bone morphogenetic proteins 1 to 7 in human breast cancer, expression pattern and clinical/prognostic relevance. *J Exp Ther Oncol* **7**, 327-38.
Dieci, M. V., Orvieto, E., Dominici, M., Conte, P. and Guarneri, V. Rare Breast Cancer Subtypes: Histological, Molecular, and Clinical Peculiarities. *The Oncologist* **19**, 805-813.
Elsamany, S. and Abdullah, S. (2014). Triple-negative breast cancer: future prospects in diagnosis and management. *Med Oncol* **31**, 834.
Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A. et al. (2008). Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* **14**, 518-527.
Finetti, P., Guille, A., Adelaide, J., Birnbaum, D., Chaffanet, M. and Bertucci, F. o. ESPL1 is a candidate oncogene of luminal B breast cancers. *Breast Cancer Research and Treatment* **147**, 51-59.
Forsman, C. L., Ng, B. C., Heinze, R. K., Kuo, C., Sergi, C., Gopalakrishnan, R., Yee, D., Graf, D., Schwertfeger, K. L. and Petryk, A. (2012). BMP-binding protein

twisted gastrulation is required in mammary gland epithelium for normal ductal elongation and myoepithelial compartmentalization. *Dev Biol* **373**, 95-106.
Friedl, P. and Gilmour, D. (2009). Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* **10**, 445-457.
Gatza, M. L., Silva, G. O., Parker, J. S., Fan, C. and Perou, C. M. An integrated genomics approach identifies drivers of proliferation in luminal-subtype human breast cancer. *Nat Genet* **advance online publication**.
Gazzerro, E. and Canalis, E. (2006). Bone morphogenetic proteins and their antagonists. *Rev Endocr Metab Disord* **7**, 51-65.
Gazzerro, E., Deregowski, V., Stadmeyer, L., Gale, N. W., Economides, A. N. and Canalis, E. (2006). Twisted gastrulation, a bone morphogenetic protein agonist/antagonist, is not required for post-natal skeletal function. *Bone* **39**, 1252-1260.
Ghosh, A., Saginc, G., Leow, S. C., Khattar, E., Shin, E. M., Yan, T. D., Wong, M., Zhang, Z., Li, G., Sung, W. K. et al. (2012). Telomerase directly regulates NF-kappaB-dependent transcription. *Nat Cell Biol* **14**, 1270-81.
Godfrey, R., Arora, D., Bauer, R., Stopp, S., Muller, J. P., Heinrich, T., Bohmer, S. A., Dagnell, M., Schnetzke, U., Scholl, S. et al. (2012). Cell transformation by FLT3 ITD in acute myeloid leukemia involves oxidative inactivation of the tumor suppressor protein-tyrosine phosphatase DEP-1/ PTPRJ. *Blood* **119**, 4499-511.
Gonzalez, D. M. and Medici, D. (2014). Signaling mechanisms of the epithelial-mesenchymal transition.
Group, U. S. C. S. W. (2014). 1999-2011 Incidence and Mortality Web-based Report., (ed.: Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute.
Hens, J. R., Dann, P., Zhang, J. P., Harris, S., Robinson, G. W. and Wysolmerski, J. (2007). BMP4 and PTHrP interact to stimulate ductal outgrowth during embryonic mammary development and to inhibit hair follicle induction. *Development* **134**, 1221-30.
Hogan, B. L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* **10**, 1580-94.
Howard, B. A. and Lu, P. (2014). Stromal regulation of embryonic and postnatal mammary epithelial development and differentiation. In *Development of Ectodermal Organs*, vol. 25â€“26 (ed., pp. 43-51.
Katsuno, Y., Hanyu, A., Kanda, H., Ishikawa, Y., Akiyama, F., Iwase, T., Ogata, E., Ehata, S., Miyazono, K. and Imamura, T. (2008). Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. *Oncogene* **27**, 6322-33.
Kaufhold, S. and Bonavida, B. (2014). Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. *Journal of Experimental & Clinical Cancer Research* **33**, 62.
Kouros-Mehr, H., Kim, J. W., Bechis, S. K. and Werb, Z. (2008). GATA-3 and the regulation of the mammary luminal cell fate. *Curr Opin Cell Biol* **20**, 164-70.

Kuhl, S. J. and Kuhl, M. (2012). On the role of Wnt/Î²-catenin signaling in stem cells. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1830**, 2297-2306.
Langenfeld, E., Deen, M., Zachariah, E. and Langenfeld, J. (2013). Small molecule antagonist of the bone morphogenetic protein type I receptors suppresses growth and expression of Id1 and Id3 in lung cancer cells expressing Oct4 or nestin. *Mol Cancer* **12**, 129.
Langenfeld, E. M., Bojnowski, J., Perone, J. and Langenfeld, J. (2005). Expression of bone morphogenetic proteins in human lung carcinomas. *Ann Thorac Surg* **80**, 1028-32.
Lewis, M. T. and Visbal, A. P. (2006). The hedgehog signaling network, mammary stem cells, and breast cancer: connections and controversies. *Ernst Schering Found Symp Proc*, 181-217.
Lind, M., Eriksen, E. F. and BÃ¼nger, C. (1996). Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. *Bone* **18**, 53-57.
Liu, F., Bloch, N., Bhushan, K. R., De Grand, A. M., Tanaka, E., Solazzo, S., Mertyna, P. M., Goldberg, N., Frangioni, J. V. and Lenkinski, R. E. (2008). Humoral bone morphogenetic protein 2 is sufficient for inducing breast cancer microcalcification. *Mol Imaging* **7**, 175-86.
Liu, W., Jeganathan, G., Amiri, S., Morgan, K., Ryan, B. and Pine, S. Asymmetric segregation of template DNA strands in basal-like human breast cancer cell lines. *Molecular Cancer* **12**, 139.
Lu, X., Mazur, S. J., Lin, T., Appella, E. and Xu, Y. (2013). The pluripotency factor nanog promotes breast cancer tumorigenesis and metastasis. *Oncogene*.
MacKenzie, B., Wolff, R., Lowe, N., Billington, C. J., Jr., Peterson, A., Schmidt, B., Graf, D., Mina, M., Gopalakrishnan, R. and Petryk, A. (2009). Twisted gastrulation limits apoptosis in the distal region of the mandibular arch in mice. *Dev Biol* **328**, 13-23.
Maemura, K., Yoshikawa, H., Yokoyama, K., Ueno, T., Kurose, H., Uchiyama, K. and Otsuki, Y. Delta-like 3 is silenced by methylation and induces apoptosis in human hepatocellular carcinoma. *Int J Oncol* **42**, 817-22.
Marazzi, G., Wang, Y. and Sassoon, D. (1997). Msx2 Is a Transcriptional Regulator in the BMP4-Mediated Programmed Cell Death Pathway. *Developmental Biology* **186**, 127-138.
Massagué, J. (1998). TGF-β Signal Transduction. *Annual Review of Biochemistry* **67**, 753-791.
Masuda, H., Otsuka, F., Matsumoto, Y., Takano, M., Miyoshi, T., Inagaki, K., Shien, T., Taira, N., Makino, H. and Doihara, H. (2011). Functional interaction of fibroblast growth factor-8, bone morphogenetic protein and estrogen receptor in breast cancer cell proliferation. *Mol Cell Endocrinol* **343**, 7-17.
McEvoy, J., Flores-Otero, J., Zhang, J., Nemeth, K., Brennan, R., Bradley, C., Krafcik, F., Rodriguez-Galindo, C., Wilson, M., Xiong, S. et al. (2011). Coexpression

of Normally Incompatible Developmental Pathways in Retinoblastoma Genesis. *Cancer Cell* **20**, 260-275.
Melnick, M., Petryk, A., Abichaker, G., Witcher, D., Person, A. D. and Jaskoll, T. (2006). Embryonic salivary gland dysmorphogenesis in Twisted gastrulation deficient mice. *Arch Oral Biol* **51**, 433-8.
Michos, O., Panman, L., Vintersten, K., Beier, K., Zeller, R. and Zuniga, A. e. (2004). Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. *Development* **131**, 3401-3410.
Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T. and Miyazono, K. (2002). Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* **7**, 1191-204.
Montesano, R. (2007). Bone morphogenetic protein-4 abrogates lumen formation by mammary epithelial cells and promotes invasive growth. *Biochem Biophys Res Commun* **353**, 817-22.
Mukhopadhyay, P., Singh, S., Greene, R. M. and Pisano, M. M. (2006). Molecular fingerprinting of BMP2- and BMP4-treated embryonic maxillary mesenchymal cells. *Orthod Craniofac Res* **9**, 93-110.
Nakayama, N., Han, C.-y. E., Scully, S., Nishinakamura, R., He, C., Zeni, L., Yamane, H., Chang, D., Yu, D., Yokota, T. et al. (2001). A Novel Chordin-like Protein Inhibitor for Bone Morphogenetic Proteins Expressed Preferentially in Mesenchymal Cell Lineages. *Developmental Biology* **232**, 372-387.
Neman, J., Choy, C., Kowolik, C. M., Anderson, A., Duenas, V. J., Waliany, S., Chen, B. T., Chen, M. Y. and Jandial, R. (2013). Co-evolution of breast-to-brain metastasis and neural progenitor cells. *Clin Exp Metastasis* **30**, 753-68.
Nishinakamura, R. and Sakaguchi, M. (2014). BMP signaling and its modifiers in kidney development. *Pediatric Nephrology* **29**, 681-686.
Nosaka, T., Morita, S., Kitamura, H., Nakajima, H., Shibata, F., Morikawa, Y., Kataoka, Y., Ebihara, Y., Kawashima, T., Itoh, T. et al. (2003). Mammalian twisted gastrulation is essential for skeleto-lymphogenesis. *Mol Cell Biol* **23**, 2969-80.
Obradovic Wagner, D., Sieber, C., Bhushan, R., Börgermann, J. H., Graf, D. and Knaus, P. (2010). BMPs: From Bone to Body Morphogenetic Proteins. *Science Signaling* **3**, mr1-mr1.
Oelgeschlager, M., Larrain, J., Geissert, D. and De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* **405**, 757-63.
Owens, P., Pickup, M. W., Novitskiy, S. V., Chytil, A., Gorska, A. E., Aakre, M. E., West, J. and Moses, H. L. (2011). Disruption of bone morphogenetic protein receptor 2 (BMPR2) in mammary tumors promotes metastases through cell autonomous and paracrine mediators. *Proc Natl Acad Sci U S A* **109**, 2814-9.
Peltoketo, H., Allinen, M., Vuosku, J., Kujala, S., Lundan, T., Salminen, A., Winqvist, R. and Vainio, S. (2004). Characterization and expression of the human

WNT4; lack of associated germline mutations in high--to moderate--risk breast and ovarian cancer. *Cancer Lett* **213**, 83-90.
Perotti, C., Karayazi, Ã., Moffat, S. and Shemanko, C. S. (2012). The bone morphogenetic protein receptor-1A pathway is required for lactogenic differentiation of mammary epithelial cells in vitro. *In Vitro Cellular & Developmental Biology - Animal* **48**, 377-384.
Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A. et al. (2000). Molecular portraits of human breast tumours. *Nature* **406**, 747-52.
Petryk, A., Anderson, R. M., Jarcho, M. P., Leaf, I., Carlson, C. S., Klingensmith, J., Shawlot, W. and O'Connor, M. B. (2004). The mammalian twisted gastrulation gene functions in foregut and craniofacial development. *Dev Biol* **267**, 374-86.
Piek, E., Moustakas, A., Kurisaki, A., Heldin, C. H. and ten Dijke, P. (1999). TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Sci* **112 (Pt 24)**, 4557-68.
Riggs, J. W., Barrilleaux, B. L., Varlakhanova, N., Bush, K. M., Chan, V. and Knoepfler, P. S. (2012). Induced pluripotency and oncogenic transformation are related processes. *Stem Cells Dev* **22**, 37-50.
Rodriguez-Martinez, A., Alarmo, E. L., Saarinen, L., Ketolainen, J., Nousiainen, K., Hautaniemi, S. and Kallioniemi, A. (2011). Analysis of BMP4 and BMP7 signaling in breast cancer cells unveils time-dependent transcription patterns and highlights a common synexpression group of genes. *BMC Med Genomics* **4**, 80.
Romero, A., Prat, A., Garcia-Saenz, J. A., Del Prado, N., Pelayo, A., Furio, V., Roman, J. M., de la Hoya, M., Diaz-Rubio, E., Perou, C. M. et al. Assignment of tumor subtype by genomic testing and pathologic-based approximations: implications on patient's management and therapy selection. *Clin Transl Oncol* **16**, 386-94.
Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 479-83.
Sailer, M. H., Gerber, A., Tostado, C., Hutter, G., Cordier, D., Mariani, L. and Ritz, M. F. (2013). Non-invasive neural stem cells become invasive in vitro by combined FGF2 and BMP4 signaling. *J Cell Sci* **126**, 3533-40.
Shebanova, O. and Hammer, D. A. (2014). Biochemical and mechanical extracellular matrix properties dictate mammary epithelial cell motility and assembly. *Biotechnology Journal* **7**, 397-408.
Shon, S. K., Kim, A., Kim, J. Y., Kim, K. I., Yang, Y. and Lim, J. S. (2009). Bone morphogenetic protein-4 induced by NDRG2 expression inhibits MMP-9 activity in breast cancer cells. *Biochem Biophys Res Commun* **385**, 198-203.
Sieber, C., Kopf, J., Hiepen, C. and Knaus, P. (2009). Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev* **20**, 343-55.

Simic, P. and Vukicevic, S. (2005). Bone morphogenetic proteins in development and homeostasis of kidney. *Cytokine Growth Factor Rev* **16**, 299-308.
Simmons, D. G. and Kennedy, T. G. (2002). Uterine Sensitization-Associated Gene-1: A Novel Gene Induced Within the Rat Endometrium at the Time of Uterine Receptivity/Sensitization for the Decidual Cell Reaction. *Biology of Reproduction* **67**, 1638-1645.
Singh, A. and Morris, R. J. (2010). The Yin and Yang of bone morphogenetic proteins in cancer. *Cytokine Growth Factor Rev* **21**, 299-313.
Sladek, N. E., Kollander, R., Sreerama, L. and Kiang, D. (2002). Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study. *Cancer Chemotherapy and Pharmacology* **49**, 309-321.
Steinert, S., Kroll, T. C., Taubert, I., Pusch, L., Hortschansky, P., Hoffken, K., Wolfl, S. and Clement, J. H. (2008). Differential expression of cancer-related genes by single and permanent exposure to bone morphogenetic protein 2. *J Cancer Res Clin Oncol* **134**, 1237-45.
Takahashi, M., Otsuka, F., Miyoshi, T., Otani, H., Goto, J., Yamashita, M., Ogura, T., Makino, H. and Doihara, H. (2008). Bone morphogenetic protein 6 (BMP6) and BMP7 inhibit estrogen-induced proliferation of breast cancer cells by suppressing p38 mitogen-activated protein kinase activation. *J Endocrinol* **199**, 445-55.
Virtanen, S., Alarmo, E.-L., SandstrÃ¶m, S., Ampuja, M. and Kallioniemi, A. (2011). Bone morphogenetic protein â^' 4 and â^' 5 in pancreatic cancerâ€"Novel bidirectional players. *Experimental Cell Research* **317**, 2136-2146.
Vogelstein, B. and Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nat Med* **10**, 789-799.
von Minckwitz, G., Untch, M. and Loibl, S. Update on neoadjuvant/preoperative therapy of breast cancer: experiences from the German Breast Group. *Curr Opin Obstet Gynecol* **25**, 66-73.
Wagner, D. O., Sieber, C., Bhushan, R., Borgermann, J. H., Graf, D. and Knaus, P. (2010). BMPs: from bone to body morphogenetic proteins. *Sci Signal* **3**, mr1.
Walsh, D. W., Godson, C., Brazil, D. P. and Martin, F. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. *Trends in Cell Biology* **20**, 244-256.
Wang, B., Miyagoe-Suzuki, Y., Yada, E., Ito, N., Nishiyama, T., Nakamura, M., Ono, Y., Motohashi, N., Segawa, M., Masuda, S. et al. (2011a). Reprogramming efficiency and quality of induced Pluripotent Stem Cells (iPSCs) generated from muscle-derived fibroblasts of mdx mice at different ages. *PLoS Curr* **3**, RRN1274.
Wang, K., Feng, H., Ren, W., Sun, X., Luo, J., Tang, M., Zhou, L., Weng, Y., He, T.-C. and Zhang, Y. (2011b). BMP9 inhibits the proliferation and invasiveness of breast cancer cells MDA-MB-231. *Journal of Cancer Research and Clinical Oncology* **137**, 1687-1696.

Wang, N. J., Sanborn, Z., Arnett, K. L., Bayston, L. J., Liao, W., Proby, C. M., Leigh, I. M., Collisson, E. A., Gordon, P. B., Jakkula, L. et al. (2011c). Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proceedings of the National Academy of Sciences* **108**, 17761-17766.
Watson, C. J. and Khaled, W. T. (2008). Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* **135**, 995-1003.
Wills, A., Harland, R. M. and Khokha, M. K. (2006). Twisted gastrulation is required for forebrain specification and cooperates with Chordin to inhibit BMP signaling during X. tropicalis gastrulation. *Dev Biol* **289**, 166-78.
Wotton, D. and Massague, J. (2001). Smad transcriptional corepressors in TGF beta family signaling. *Curr Top Microbiol Immunol* **254**, 145-64.
Xia, L., Zheng, X., Zheng, W., Zhang, G., Wang, H., Tao, Y. and Chen, D. (2012). The Niche-Dependent Feedback Loop Generates a BMP Activity Gradient to Determine the Germline Stem Cell Fate. *Current Biology* **22**, 515-521.
Ye, L., Bokobza, S. M. and Jiang, W. G. (2009). Bone morphogenetic proteins in development and progression of breast cancer and therapeutic potential (review). *Int J Mol Med* **24**, 591-7.
Ying, Q.-L., Nichols, J., Chambers, I. and Smith, A. (2003). BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. *Cell* **115**, 281-292.
Zakin, L. and De Robertis, E. M. (2004). Inactivation of mouse Twisted gastrulation reveals its role in promoting Bmp4 activity during forebrain development. *Development* **131**, 413-24.
Zuniga, A., Haramis, A.-P. G., McMahon, A. P. and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401**, 598-602.