Embryonic Morphogen Nodal Promotes Breast Cancer Growth and Progression

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Abstract

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

Introduction

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

In addition to enhanced proliferation and evasion of apoptosis during cancer progression, aberrant expression of stem cell factors within breast tumours sustains aggressive phenotypes, and is associated with growth-promoting profiles in tumour cells and their microenvironments. One example of a stem cell factor that is associated with cancer progression is Nodal, an embryonic morphogen and member of the Transforming Growth Factor-Beta (TGF-β) superfamily. Nodal expression is limited to pluripotent stem cells during embryonic development and to specialized dynamic adult tissue (such as the cycling endometrium), but is re-expressed to induce growth programmes in cancers such as melanoma, prostate cancer, endometrial cancer, glioma, pancreatic cancer and hepatocellular carcinoma [7–15]. In accordance with its documented contribution to tumour growth, Nodal has recently been linked to proliferation in a variety of normal physiological systems. For example, Harrison and colleagues have studied Nodal signalling in human endometrium during the various phases of remodelling, and found that Nodal is highly expressed throughout the proliferative and early secretory phases, and is abruptly downregulated by the mid-secretory phase.

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Nodal mediates growth of breast cancer cell lines

Methods

Cell Lines and Treatments

Two well-differentiated, breast cancer cell lines (MCF-7 and T47D) and three poorly-differentiated, cell lines (MDA-MB-231, MDA-MB-468 and Hs578t) [20] were used. All cancer cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained as per instructions. The phenotypes of these cells were verified by ATCC in accordance with protocols available on the ATCC website. To increase Nodal signalling, we used a Nodal expression vector (versus an empty pcDNA3.3 vector; pcDNA13.5-3-TOPO® cloning kit; Invitrogen) as previously described [21]. We also employed recombiant human Nodal (rhNodal; R&D). To decrease Nodal signalling, we used Nodal-targeted shRNAs (versus scrambled control shRNAs) as previously described [21]. Two Nodal-targeted shRNAs were used, a HuSH-29mer (Id: G31171; OriGene) and a GIPZ lentiviral shRNAmir (Id: V2LHS_155453; Open Biosystems) to rule-out off-target effects. Transfection was performed with Arrest-In (Open Biosystems) or Lipofectamine (Invitrogen) as per manufacturer instructions. For stable selection, Puromycin (200–500 μg/mL) or Genticin (200–500 μg/mL) was used. To inhibit Nodal signalling, we also used SB431542. SB431542 selectively inhibits Activin, TGF-β and Nodal signalling but not BMP signalling. In addition, SB431542 does not affect components of the ERK, JNK, or p38 MAP kinase pathways [22].

RNA Extraction and RT-PCR

RNA isolation was performed using the Perfect Pure RNA cultured cell kit (5 Prime), and DNase was used to degrade genomic DNA. Reverse transcription was performed using 2 μg of RNA and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed with TaqMan® gene expression human primer/probe sets. For a list of primer/probes, see Table S1. For analysis of BAX and BCL2 gene expression in response to treatments, Ct values were normalized to HPRT1, and compared using the ΔΔCt method. Variability in housekeeping gene expression across cell lines confounded results obtained with real time-RT-PCR. Hence, semi-quantitative RT-PCR was used to measure Nodal receptor components across breast cancer cell lines using H9 hESC mRNA as a positive control. For semiquantitative PCR, 1/20th of the cDNA reaction was used as template for amplification using AmpliTag Gold® 360 Master Mix (Applied Biosystems). Validated primer probes were used as described above. The loading control HPRT1 was amplified with forward primer: 5'-agtcggacctgccctgg-3', and reverse primer: 5'-ctgggtagatgcactgtcaggcag-3'. The following cycling conditions were used: 95°C for 30 sec, 55°C (ALK4, ALK7, CRIPTO) or 64°C (HPRT1) for 30 sec, and 72°C for 1 min. ALK4 and HPRT1 were subjected to 30 cycles. ALK7 and CRIPTO were subjected to 35 cycles.

Western Blotting

Protein lysates were prepared using Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific) and Halt Protease Inhibitor Cocktail (Thermo Scientific) as per manufacturer’s instructions. Equal amounts of protein were reduced and separated by SDS-polyacrylamide gel electrophoresis, and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in 5% milk, incubated with primary antibody, washed, and incubated with horseradish peroxidase-labelled secondary antibody. For a list of primary antibodies see Table S2. Secondary antibodies were detected by enhanced chemiluminescence (Super Signal; Pierce). In accordance with previous studies [9,11,23,24], three banding locations were detected for Nodal: Pro-Nodal at ~39 kDa, pre-pro-Nodal at ~50 kDa, and mature Nodal at ~15 kDa. The 30 kDa species is highly variable due to differences in post-translational modifications and protein lystate handling, and the 15 kDa band is poorly abundant in protein lystate. For consistency, we used the 39 kDa band to assess Nodal expression in lysates and the 15 kDa band in conditioned medium, as previously described [21,24].

Tumour Assays in Nude Mice

All experiments involving animals were approved by the Animal Use Subcommittee at the University of Western Ontario.

Nodal loss-of-function flank tumour assay. MDA-MB-468 cells were transfected with a Control HuSH shRNA, or a Nodal-targeted HuSH shRNA. 2,500,000 cells in 100 μL of RPMI+Matrigel (1:1) were injected into the right flank of 6-8 week old athymic Nude-Foxn1nu mice. Tumour measurements were taken twice per week and a digital caliper was used to measure Length × Width × Depth of the tumour upon excision in order to calculate volume.

Nodal loss-of-function orthotopic tumour assay. MDA-MB-231 cells were transfected with a Control GIPZ shRNA, or a Nodal-targeted GIPZ shRNA. 500,000 cells in 50 μL of RPMI
were injected into the mammary fat pad via the nipples of 6–8 week old athymic Nude-Foxn1nu mice. Tumour measurements were taken as described above.

In vitro Cell Growth Curves

Cells were seeded into 6-well plates (100,000 cells/well) and counted over 4 days. Media containing dead and alive cells was collected. Attached cells were harvested using Trypsin, combined with media, spun down, and resuspended with Trypan Blue. A Countess automated cell counter (Invitrogen) was used to calculate total cell number, live cells, dead cells, and viability.

Cell Trace Violet Proliferation Assays

Cells were starved 21–22 hours and then labelled with 2.5 μM (T47D lines) or 5 μM (MDA-MB-231 lines) Cell Trace Violet (CTV, Invitrogen) as per the manufacturer’s instructions. Briefly, culture medium was removed from cells and replaced with CTV diluted to 2.5 or 5 μM in pre-warmed phosphate-buffered saline (PBS). Cells were incubated 20 min at 37°C after which CTV solution was removed and cells were washed twice with pre-warmed complete medium and then left in fresh medium for 4–6 days. Cells were harvested via trypsinization and then washed in FACS buffer (PBS+1% FBS+2 mM EDTA) before flow cytometric acquisition. Fluorescence-activated cell sorting (FACS) was performed on an LSRII (Becton Dickinson, Mississauga ON) calibrated with CaliBRITE Beads (Becton Dickerson, Mississauga ON). Live cell singlets were gated based on forward and side-scatter properties. Analysis was performed using FlowJo® software (Tree Star, Ashland, OR, USA, Version 9.5.2).

TUNEL Staining

Cells were grown on glass coverslips to ~50% confluence and then fixed with 4% paraformaldehyde. The DeadEnd colorimetric TUNEL system (Promega) was used to measure apoptosis as per instructions. The % TUNEL positive cells were determined by counting the number of positive cells in 3 fields of view on each slide taken at 40X. This number was divided by the total number of cells in these fields. At least 4 slides were used per experimental group.

Immuonofluorescence

Cells were fixed with 4% paraformaldehyde, made permeable with 20 mM Heps, 0.5% TritonX-100 and blocked with serum-free protein block (DAKO). Primary antibodies were diluted in antibody diluent (DAKO) to the concentrations outlined in Table S2, and appropriate fluorochrome-conjugated secondary antibodies were used according to manufacturer recommendations. Nuclei were stained with DAPI (0.1 mg/mL; Invitrogen/Molecular Probes, Eugene, OR), and images were obtained using confocal microscopy (Zeiss 510 META, Carl Zeiss Inc.).

Experimental Metastasis Assay in NOD/SCID/MPSVII Mice

500,000 cells in 700 μL Ca2+-free HBSS were injected into the tail vein of NOD/SCID/MPSVII mice. Mice were sacrificed at 4 weeks (to assess micrometastases) and 8 weeks (to assess macrometastases). Lung, brain, and liver from transplanted NOD/SCID/MPSVII mice were frozen in OCT embedding medium (Sakura Finetek, Torrance, CA) for histochemical analysis. Serial sections at 10-μm thickness, were fixed in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO), and blocked with mouse-on-mouse reagent (Vector Laboratories, Burlingame, CA). Sections were analyzed for human cells by colourimetric detection of ubiquitous GUSB activity in human cells as previously described using napthol AS-BI β-D-glucuronide (Sigma-Aldrich) substrate [23], and counterstained with haematoxylin. Metastases that were<100 cells were considered ‘micro’, while metastases that were>100 cells were considered ‘macro’. For each mouse organ, 5–6 sections were acquired from evenly spaced areas through the tissue, and the average number of metastases per mouse organ was calculated. The proliferation-to-apoptosis ratio was determined by counting Ki67 and TUNEL positive nuclei in matched serial sections. Briefly, tissues were formalin-fixed and paraffin-embedded and immunohistochemical staining on this tissue was conducted using a human-specific Ki67 antibody (Table S2) as per manufacturer suggestion. The DeadEnd colorimetric TUNEL system (Promega) was used to measure apoptosis as per instructions. The proliferation-to-apoptosis ratio was determined by counting Ki67 and TUNEL positive nuclei in matched serial sections. At least 3 pairs of serial sections, evenly spaced through the tissue, were averaged per mouse to yield an average proliferation-to-apoptosis score for that animal.

Statistical Analyses

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

Results and Discussion

Expression of Nodal, ALK4, ALK7 and Cripto in Breast Cancer Cell Lines

Through Western blot analyses, we determined that Nodal protein is elevated in cell lysates and in conditioned media from poorly-differentiated Hs578t, MDA-MB-231 and MDA-MB-468 breast cancer cell lines compared to well-differentiated MCF-7 and T47D cell lines (Fig 1A,B). This is consistent with previous reports that show high Nodal expression in aggressive melanoma, prostate, and breast cancer cell lines compared to poorly aggressive lines [7,9,11,18].

Nodal signals through interactions with Cripto-1 and the Activin-Like Kinase type I (ALK4/7) and type II (ActRIIB) receptor complex. Activation of this receptor complex leads to SMAD2/3 phosphorylation, and subsequent induction of Nodal-dependent gene expression [26]. It has been reported that Nodal receptor components are expressed at varying levels in prostate cancer cell lines [27]. In order to ensure the breast cancer cell lines used in this study have the potential to respond to alterations in Nodal, we measured the expression of the members of the Nodal receptor complex. Accordingly, we determined that ALK and Cripto protein are present in Hs578t, MDA-MB-231, MDA-MB-468, T47D and MCF-7 breast cancer cell lines at varying levels and that all of these cell lines express ALK4, ALK7 and Cripto mRNA (Fig 1C,D). This suggests that these cell lines are able to respond to and carry out Nodal-induced signal transduction. Since they secrete higher levels of Nodal, we decided to use MDA-MB-468 and MDA-MB-231 cells in our loss-of-function models, and because they secrete very low levels of Nodal, but still express Nodal receptors, we chose to use T47D cells to study how the upregulation of Nodal expression affects breast cancer cells.
Nodal Knockdown Prevents Tumourigenesis using Aggressive Breast Cancer Cells

Given that Nodal is associated with aggressive cancers and breast cancer cell lines, we first sought to determine whether stable Nodal knockdown regulates breast cancer tumourigenesis in vivo. Previous studies demonstrated that transient inhibition of Nodal with Morpholinos or exposure to its antagonist, Lefty, diminished tumour initiation in breast cancer and melanoma models [9,11]. In order to better understand the role of Nodal in tumour growth over an extended period of time, we stably knocked down Nodal expression in human breast cancer cell lines using puromycin-selectable shRNAs. In our first model, we injected 2.5 million MDA-MB-468 cells transfected with a Control shRNA (468+shControl) or a Nodal-targeted shRNA (468+shNodal) into the flanks of nude mice, and measured tumour growth over 6 weeks. This approach revealed that Nodal knockdown significantly impaired MDA-MB-468 tumour growth, with a 2-fold reduction in tumour volume following excision (p<0.05) (Fig. 2A–C).

As a corollary to this experiment, in our second model, we injected 500,000 MDA-MB-231 cells transfected with a Control shRNA (231+shControl) or Nodal-targeted shRNA (231+shNodal) through the nipple into the mammary fat pad of nude mice, and measured tumour growth over 9 weeks. Compared to our flank model, this model was more stringent since we injected fewer cells (0.5 versus 2.5 million), Matrigel was not used to help tumours grow over time, the 231+shNodal tumours experienced a plateau in growth at a diameter of approximately 1.5 mm. This suggested to us that Nodal inhibition may alter proliferation and cell death ratios to counteract tumour growth.

Loss of Nodal Expression Reduces Proliferation and Increases Apoptotic Phenotypes in Aggressive MDA-MB-231 Breast Cancer Cells

Given our finding that Nodal inhibition causes a reduction in tumour growth in vivo, we examined the effects of Nodal knockdown on cell proliferation in vitro using Trypan Blue exclusion assays. As a Nodal loss-of-function model, we compared growth curves for 231+shNodal cells versus 231+shControl cells. We found that unlike 231+shControl tumours which continued to grow over time, the 231+shNodal tumours experienced a plateau in growth at a diameter of approximately 1.5 mm. This suggested to us that Nodal inhibition may alter proliferation and cell death ratios to counteract tumour growth.

Volume following excision (p<0.05) (Fig. 2F). Importantly, we observed a phenomenon that was not apparent in our flank model. We found that unlike 231+shControl tumours which continued to grow over time, the 231+shNodal tumours experienced a plateau in growth at a diameter of approximately 1.5 mm. This suggested to us that Nodal inhibition may alter proliferation and cell death ratios to counteract tumour growth.

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indictative of reduced mitosis (Fig. 3C). Taken together, Nodal loss-of-function decreased proliferation; however the changes were small, suggesting that alterations in proliferation did not solely account for the robust effects seen on tumour growth in vivo.

Given Nodal had a small effect on proliferation in vitro, we hypothesized that perhaps Nodal could also regulate apoptotic phenotypes, which might help to explain the large differences observed during in vivo tumour growth. In order to explore the role of Nodal in the regulation of apoptosis, we first examined the effects of knocking Nodal down on the percentage of TUNEL positive (apoptotic) cells in vitro. Accordingly, Nodal knockdown in MDA-MB-231 cells resulted in a 3.5% increase (from 2.4 to 5.9%) in the number of apoptotic cells as compared to control conditions (n = 24, p = 0.001) (Fig. 3D). Furthermore, Western blot analyses for activated (cleaved) caspase-9 in 231+shNodal cells versus 231+shControl cells revealed that cleaved caspase-9 is increased in response to Nodal knockdown, indicative of elevated levels of apoptosis (Fig. 3E). Given that caspase-9 is frequently associated with mitochondria-mediated apoptosis, we quantified BAX and BCL2 mRNA expression in 231+Control cells versus 231+shNodal cells, since these factors also play a role in mitochondria-mediated apoptosis. Real time RT-PCR analysis indicated that there was a significant increase in BAX expression (n = 4, p = 0.029) and a significant decrease in BCL2 expression (n = 4, p = 0.029) in 231+shNodal cells compared to controls (Fig. 3F, G).

Gain of Nodal Expression Increases Proliferation and Decreases Apoptotic Phenotypes in Poorly Aggressive T47D Breast Cancer Cells

As an extension to our loss-of-function results, we next examined the effects of over-expressing Nodal in poorly aggressive T47D breast cancer cells (T47D+Nodal), which do not normally express high levels of this morphogen, versus transfection with an empty vector control (T47D+EV). Using a trypan blue exclusion assay, we found that T47D+Nodal cells displayed a significant increase in proliferation after 3 days compared to T47D+EV cells (n = 3, p = 0.046) (Fig. 4A). Likewise, using the CTV assay in three independent experiments, we determined that T47D+Nodal cells proliferated more than parental T47D+EV cells as evidenced by lower CTV mfi (Fig. 4B). In the representative example shown, CTV mfi was 2738 for T47D+Nodal compared to 3319 for T47D+EV cells six days post-synchronization. When Western blot analysis was performed to evaluate the phosphorylation status of Histone H3, we found that T47D+Nodal cells displayed elevated P-Histone H3 at four different sites (including Ser10, Ser28, Thr3, and Thr11) compared to T47D+EV cells, indicative of elevated mitosis (Fig. 4C). Of note, using a fluorescence-based Live-Dead assay, in a previous study we did not see major changes in cell viability in T47D+Nodal cells relative to T47D+EV cells [21]. This was likely because the Live/Dead assay was less sensitive than the multiple assays employed here.

To determine the effects of Nodal on apoptosis, we first conducted TUNEL staining on cells grown in vitro. In accordance
Figure 3. Nodal knockdown decreases proliferation and increases apoptosis in aggressive MDA-MB-231 breast cancer cells. (A) Trypan Blue exclusion was used to count live cells daily to generate growth curves over 3 days, in response to altered Nodal expression. MDA-MB-231 cells transfected with a Nodal-targeted shRNA (231+shNodal) exhibited a significant decrease in proliferation over 3 days compared to cells transfected with a scrambled Control shRNA (231+shControl) (n = 3; p = 0.047). (B) Representative histogram of mean fluorescence intensity (mfi) in 231+shNodal and 231+shControl cells labelled with Cell Trace Violet (CTV) for 4 days after synchronization via serum starvation. There was a greater loss of CTV in 231+shControl compared to 231+shNodal cells (4801 and 6006, respectively) indicating that control cells proliferated more than their shNodal-treated counterparts. (C) Western blots demonstrating decreased phosphorylated histone H3 at 4 different sites, including Thr11, Ser10, Ser28 and Thr3 in 231+shNodal cells compared to 231+shControl cells. Total histone H3 and β-Actin are used as controls. (D) Representative images of TUNEL staining and corresponding quantification of percent TUNEL-positive cells in 231+shNodal and 231+shControl cells grown in vitro. TUNEL positive cells are delineated by arrows and nuclei are counterstained blue. Micron bars equal 25 μm. The percentage of TUNEL positive cells is significantly higher in 231+shNodal cells as compared to 231+shControl cells (n = 23; p = 0.001). (E) Western blot demonstrating that cleavage of caspase-9 is elevated in 231+shNodal compared to 231+shControl cells. Uncleaved caspase-9 and β-Actin are used as controls. (F) Real time RT-PCR analysis demonstrating that BAX mRNA expression is significantly higher in 231+shNodal compared to 231+shControl cells (n = 4, p = 0.029). (G) Real time RT-PCR analysis demonstrating that BCL2 mRNA expression is significantly lower in 231+shNodal compared to 231+shControl cells (n = 4, p = 0.029). All bar graphs are presented as mean ± S.E.M. for replicate values. Asterisks indicate a significant difference as specified compared to controls.
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Figure 4. Nodal over-expression increases proliferation and decreases apoptosis in poorly aggressive T47D breast cancer cells. (A) Western blot demonstrating that Nodal protein is elevated in T47D cells transfected with a Nodal expression construct (T47D+Nodal) compared to empty vector controls (T47D+EV). The ~39 kDa Pro-Nodal band is presented and Actin is used as a loading control. Trypan Blue exclusion was used to count live cells daily to generate growth curves over 3 days, in response to altered Nodal expression. T47D+Nodal cells exhibited a significant increase in proliferation compared to T47D+EV cells over 3 days (n = 3, p = 0.046). (B) Representative histogram of mfi in T47D+EV and T47D+Nodal cells labelled with CTV for 6 days after synchronization via serum starvation. There was a greater loss of CTV in T47D+Nodal compared to T47D+EV cells (2738 and 3319, respectively) indicating that proliferation increased with Nodal over-expression. (C) Western blots demonstrating increased phosphorylated histone H3 at 4 different sites, including Thr11, Ser10, Ser28 and Thr3 in T47D+Nodal cells compared to T47D+EV cells. Total histone H3 and β-Actin are used as controls. (D) Representative images of TUNEL staining and corresponding quantification of percent TUNEL-positive cells in T47D+EVD+Nodal and T47D+EV cells. TUNEL positive cells are delineated by arrows and nuclei are counterstained blue. Micron bars equal 25 μm. The percentage of TUNEL positive cells is significantly higher in T47D+Nodal cells as compared to T47D+EV cells (n = 8; p = 0.006). (E) Western blot demonstrating that cleavage of caspase-9 is reduced in T47D+Nodal cells compared to T47D+EV cells. Uncleaved caspase-9 and β-Actin are used as controls. (F) Real time RT-PCR analysis demonstrating that BAX mRNA expression is significantly lower in T47D+Nodal cells compared to T47D+EV cells.
with our loss-of-function data, Nodal over-expression in T47D cells resulted in a 3.2% decrease (from 5.5 to 2.3%) in the number of apoptotic cells as compared to control conditions (n = 8, p = 0.006) (Fig. 4D). In order to explore the effects of Nodal on apoptosis, we performed Western blot analyses for activated (cleaved) caspase-9 in T47D+EV cells versus T47D+Nodal cells. We found that cleaved caspase-9 was present at lower levels in T47D+Nodal cells compared controls, indicative of reduced apoptosis in the presence of Nodal (Fig. 4E). Accordingly, real-time RT-PCR analysis indicated that there was a significant decrease in BAX expression (n = 5, p = 0.016) and a significant increase in BCL2 expression (n = 5, p = 0.016) in T47D+Nodal cells compared to controls (Fig. 4F,G).

Taken together, our results suggest that Nodal promotes the net growth of breast cancer cells in culture by increasing proliferation and decreasing apoptosis. This in part explains the observation that Nodal inhibition blunts tumour growth in vivo. Interestingly, it has been reported that Notch4, which regulates Nodal expression in melanoma models, promotes proliferation and inhibits apoptosis in C8161, MV3, and SK-MEL-28 melanoma cell lines [28]. A recent study also demonstrated that blocking Nodal signaling with a function-blocking antibody decreases proliferation and increases apoptosis of MDA-MB-231 and MDA-MB-468 cells in vitro [18], supporting the results presented here. Furthermore, Nodal over-expression in GBM glioma cells causes an increase in proliferation concomitant with elevated tumourigenesis in mice [8]. In contrast to the results shown here, it has been reported that over-expression of Nodal promotes apoptosis and inhibits proliferation in MDA-MB-231 breast cancer cells lines [29,30]. However, one key difference in this study is that Nodal was overexpressed in MDA-MB-231 cells (which we have shown express high endogenous Nodal), whereas in the current study, Nodal was inhibited in MDA-MB-231 cells. Furthermore, the dose of recombinant mouse Nodal that was used in the previous study was 500 ng/mL, which is 5-fold higher than the dose of recombinant human Nodal used in this investigation [30]. This brings light to the possibility that Nodal exhibits a concentration-dependent bi-phasic effect on breast cancer progression, similar to the function of TGF-β [31,32].

Inhibition of ALK4/7 (Nodal Type I Receptor) Reduces Proliferation and Increases Apoptotic Phenotypes in Highly Aggressive Breast Cancer Cell Lines

Nodal’s canonical signalling pathway includes binding to a receptor complex (including ALK4/7, ActRIIB, and Cripto) to activate phosphorylation of SMAD2/3, which translocates to the nucleus with SMAD4 to regulate gene transcription. To determine whether this receptor complex is important for cellular growth, we used a small molecule inhibitor, SB431542, which blocks the Nodal type 1 receptor (ALK4/7), and evaluated the effects of rhNodal treatment on cellular growth, and BAX and BCL2 expression. We first validated that treatment with SB431542 reduced phosphorylation of SMAD2 in MDA-MB-231 cell lines (which express high levels of Nodal) (Fig. 5A). We also performed immunofluorescence for SMAD2/3 in MDA-MB-231 and MDA-MB-468 cell lines, to validate that treatment with SB431542 caused reduced nuclear translocation of SMAD2/3, which is indicative of decreased SMAD activation (Fig. 5B). We found that treatment of both cell lines with 10 μM SB431542 caused reduced cellular growth over the course of 4 days, and this effect was not rescued by addition of rhNodal (Fig. 5C,D), suggesting that Nodal needs to activate the ALK4/7 receptor to increase proliferation. Furthermore, we found that treatment of MDA-MB-231 cells with 10 μM SB431542 caused a significant increase in BAX expression (n = 3, p = 0.002), and a significant decrease in BCL2 expression (n = 3, p = 0.022), indicative of elevated apoptosis compared to vehicle controls (Fig. 5E,F). Similarly, treatment of MDA-MB-468 cells with 10 μM SB431542 caused a significant increase in BAX expression (n = 3, p = 0.020), and a significant decrease in BCL2 expression (n = 3, p = 0.046) (Fig. 5G,H). Addition of rhNodal to SB431542-treated MDA-MB-231 cells or MDA-MB-468 cells did not rescue BAX or BCL2 expression in either cell line, indicating that regulation of BAX and BCL2 gene expression is dependent on activation of the Nodal type 1 receptor (Fig. 5E-H).

To our knowledge, no other receptors (besides the ALK4/7/ActRIIB/Cripto receptor complex) have been shown to interact with Nodal; therefore, we suspect that Nodal mediates its mitogenic effects through its canonical SMAD pathway. However, it remains elusive whether Nodal can elicit its effects through a non-canonical mechanism. For example, we have shown that Nodal is capable of mediating phosphorylation of ERK1/2 to promote invasive phenotypes in breast cancer and choriocarcinoma cell lines. Given that ERK signalling is frequently associated with elevated mitogenic activity in cancer, studies geared towards exploring whether ERK signalling is involved in the Nodal-induced phenotypes presented here would be of interest to pursue in the future. Finally, it should be noted that SB431542 does not selectively inhibit Nodal signalling: It can also block Activin and TGF-β induced responses [22]. Hence it is also possible, that Nodal mediates its effects on proliferation by regulating these pathways.

Nodal Promotes Growth from Micro to Macrometastases

Cancer becomes a fatal disease once it has metastasized and grown into a sufficient secondary tumour mass. However, the metastatic cascade is a highly inefficient process overall, and it has been reported that one of the most inefficient steps is growth at the secondary site [33,34]. Given that Nodal inhibition causes a plateau in primary tumour growth in vivo, and that it alters proliferation and cell death in vitro, we opted to test the effect of Nodal inhibition on secondary tumour growth.

Accordingly, we developed a model that takes advantage of a simple experimental metastasis assay using beta-glucuronidase (GUSB)-deficient NOD/SCID/mucopolysaccharidosis type VII (NOD/SCID) mice [25]. The GUSB model allowed us to attain single-cell resolution of transplanted human tumour cells by virtue of their constitutive GUSB activity within the GUSB-deficient mouse, thereby enabling the identification of lesions down to single-cell level that would be undetectable using conventional histology. We sacrificed the mice at two different time points, at 4 weeks and 8 weeks, following tail vein injection of 231+shControl or 231+shNodal cells. At both 4 and 8 weeks, brain and liver tissue were also evaluated for evidence of metastasis. Using this high-resolution experimental metastasis model, we found only micrometastases of approximately 1–100 cells at 4 weeks post-injection, and discovered that Nodal knockdown did not cause a significant change in the number of micrometastases that formed in the lung.
This suggested that Nodal does not affect seeding at secondary sites.

In addition to seeding, another important step in the metastatic cascade is growth at the secondary site. Indeed, metastatic tumour cells can reside and survive at secondary sites in the body while circumventing a need for growth or progression; an aspect of carcinogenesis called tumour dormancy. Tumour mass dormancy, in particular, refers to metastases that remain asymptomatic due to...
an inability to expand in size, and is often attributed to a counterbalance of proliferation and apoptosis (reviewed in [35]). Dormant tumours are often not dangerous; however, their potential to overcome their dormant state poses a threat to patient health. Accordingly, we measured proliferation-to-apoptosis ratios in the 4-week micro-lesions via immunohistochemical staining for Ki67:TUNEL. We found that 231+shControl lesions had a proliferation-to-apoptosis ratio greater than 1, indicating a potential for tumour growth, whereas 231+shNodal lesions had a proliferation-to-apoptosis ratio less than 1, indicating a state of tumour mass dormancy or regression (Fig. 6C,D).

Given these results, we expected that tumours that exhibited a potential for growth at 4 weeks would progress to macrometastases by 8 weeks, and far exceed the 100-cell limit observed in the 4-week lesions. Of note, by 8 weeks the 231+shControl lesions formed overt pulmonary metastases in all of the mice injected with these cells (Fig. 7A,B). In contrast, the 231+shNodal cells did not form macrometastases. Rather, at 8 weeks there was a significant accumulation of micrometastases in the lung compared to 231+shNodal cells at the 4 week time point (n=8, p<0.05) (Fig. 7C). We also detected metastases in the brain of 1/5 231+shControl injected mice at 4 weeks and in the liver of 1/4 231+shControl injected mice at 8 weeks. However, metastases to the brain or the liver were not detected in any of the 16 231+shNodal-injected mice (Fig. S1A,B).

Taken together, the results from our experimental metastasis assay illustrate the importance of Nodal in regulating the transition between micrometastatic and macrometastatic growth, in part...
through its ability to alter proliferation-to-apoptosis ratios necessary for normal tissue homeostasis. Similar phenomena have been reported in C57BL6/J mouse models of Lewis lung carcinoma mice, whereby poor tumour vascularization caused tumour dormancy marked by equal rates of mitosis and apoptosis [36]. Interestingly, previous findings from our laboratory have implicated Nodal in regulating breast cancer angiogenesis both in vitro and in vivo [21]. Thus in addition to directly regulating cell proliferation and/or apoptosis, Nodal may indirectly promote tumour growth by facilitating vascular recruitment. Finally, although Nodal did not affect seeding of the cells at the secondary site, micrometastases accumulated over time with Nodal-deficient cells. Conventional tumour growth assays using histology and/or whole animal imaging do not permit the single cell resolution that we obtained with the GUSB model; hence, studies done using conventional methodologies may inadvertently overlook the seeding phenomenon uncovered here.

Conclusions

Collectively, this study indicates that the stem cell-associated protein Nodal promotes breast cancer tumour growth at both primary and secondary tumour sites, by altering the balance between proliferation and apoptosis. Our results provide mechanistic insight into studies that demonstrate that cancer cells manifesting stem-cell like properties exhibit accelerated cancer growth and progression in vivo, compared to well-differentiated counterparts [37,38]. Since Nodal expression is limited to embryonic contexts, our discovery suggests a novel role for Nodal as a tumour-specific target against breast cancer progression, and for maintenance of tumour dormancy following metastatic spread.

Supporting Information

Figure 7 Nodal knockdown mitigates progression to macrometastases. (A) H&E staining demonstrates macrometastasis formation after 8 weeks post-intravenous injection in NOD/SCID/MPSVII mice for 231+shControl cells, but not 231+shNodal cells. 100% of lungs seeded with 231+shControl cells (4/4) contained macroscopic lesions at 8 weeks, whereas 0/8 lungs seeded with 231+shNodal cells contained macroscopic lesions. (B) GUSB staining to confirm human origin of lesions showing 231+shControl macrometastasis formation after 8 weeks. Although macrometastases were not detected in mice injected with 231+shNodal cells, micrometastases were detected with GUSB staining. (C) The number of 231+shNodal micrometastases that formed after 8 weeks in NOD/SCID/MPSVII mice was significantly higher than those that formed after 4 weeks (n=8, p<0.05). Bars represent the average mean number of micrometastases per section per mouse ± S.E.M.

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Figure S1 Nodal supports tumour metastasis. (A) GUSB staining of a brain metastasis from MDA-MB-231 cells transfected with a Control shRNA (231+shControl) in NOD/SCID/MPSVII mice 4 weeks post-intravenous injection (red). (B) GUSB staining demonstrates a liver macrometastasis from 231+shControl cells at 8 weeks. No tumours were found in either brain or liver from 231+shNodal cells at either 4 or 8 weeks.

Table S1

(PDF)

Table S2

(PDF)

Acknowledgments

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Author Contributions
Conceived and designed the experiments: DFQ GZ LAW DAH LMP. Performed the experiments: DFQ GZ LAW GMS DZD SDF HB DMP.

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