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REPORT

Nodal expression in triple-negative breast cancer: Cellular effects of its inhibition following doxorubicin treatment

Thomas M. Bodenstine[a,†], Grace S. Chandler[a], David W. Reed[a], Naira V. Margaryan[a], Alina Gilgur[a], Janis Atkinson[b], Nida Ahmed[b], Matthew Hyser[b], Elisabeth A. Seftor[a], Luigi Strizzi[a,c], and Mary J. C. Hendrix[a,d]

[a]Cancer Biology and Epigenomics Program at the Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA; [b]Presence Saint Francis Hospital, Evanston, IL, USA; [c]Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA; [d]Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

ABSTRACT

Triple-negative breast cancer (TNBC) represents an aggressive cancer subtype characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The independence of TNBC from these growth promoting factors eliminates the efficacy of therapies which specifically target them, and limits TNBC patients to traditional systemic neo/adjuvant chemotherapy. To better understand the growth advantage of TNBC – in the absence of ER, PR and HER2, we focused on the embryonic morphogen Nodal (associated with the cancer stem cell phenotype), which is re-expressed in aggressive breast cancers. Most notably, our previous data demonstrated that inhibition of Nodal signaling in breast cancer cells reduces their tumorigenic capacity. Furthermore, inhibiting Nodal in other cancers has resulted in improved effects of chemotherapy, although the mechanisms for this remain unknown. Thus, we hypothesized that targeting Nodal in TNBC cells in combination with conventional chemotherapy may improve efficacy and represent a potential new strategy. Our preliminary data demonstrate that Nodal is highly expressed in TNBC when compared to invasive hormone receptor positive samples. Treatment of Nodal expressing TNBC cell lines with a neutralizing anti-Nodal antibody reduces the viability of cells that had previously survived treatment with the anthracycline doxorubicin. We show that inhibiting Nodal may alter response mechanisms employed by cancer cells undergoing DNA damage. These data suggest that development of therapies which target Nodal in TNBC may lead to additional treatment options in conjunction with chemotherapy regimens – by altering signaling pathways critical to cellular survival.

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Introduction

From a clinical perspective endocrine therapies and monoclonal antibodies have been deployed for the successful treatment of breast cancers responsive to signals from the estrogen receptor (ER), progesterone receptor (PR) and/or human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC), however, exhibits little to no expression of these molecules.[1] Hence, treatment approaches for this aggressive disease remain challenging and few established targeted therapies for TNBC have been developed.[2-4] A distinct complication of TNBC relates to patients who relapse with metastatic disease following treatment. These patients face shorter survival times when compared to other breast cancer subtypes and therapy responses at this stage are poor.[5,6] Thus, identification of novel targetable molecules which drive TNBC growth are of great interest for therapeutic development.

A promising new targetable molecule unique to aggressive cancers is Nodal, a member of the transforming growth factor β (TGFβ) superfamily, critical during embryogenesis to coordinate processes such as tissue organization, body axis specification, and induction of the primary germ layers through activation of mechanisms which include the epithelial-to-mesenchymal transition (EMT).[7,8] Nodal also plays a required role in maintaining pluripotency of human embryonic stem cells.[9] While absent in most adult somatic cells, the re-expression of Nodal has been linked to numerous cancers and correlates with aggressiveness and the cancer stem cell phenotype.[10-12] Nodal has previously been shown to hold promise as a predictive and prognostic biomarker in breast cancer independent of ER, PR and HER2 status.[13] The inhibition of Nodal signaling in multiple cancer models leads to decreases in aggressive attributes such as tumorigenicity, metastasis, invasion, angiogenesis, and the plastic stem cell phenotype.[10,14] Of particular note, studies in pancreatic carcinoma and melanoma have revealed that the inhibition of Nodal signaling improves the effects of chemotherapy, suggesting additional clinical utility.[15,16] In this study, we demonstrate that Nodal is a highly expressed protein in TNBC when directly compared to hormone receptor (HR) and HER2 positive invasive breast cancer. Inhibiting Nodal signaling in TNBC cell lines following treatment with the anthracycline doxorubicin reduces viability of these cells and alters a critical signaling pathway

CONTACT Mary J. C. Hendrix mhendrix@shepherd.edu Shepherd University, P.O. Box 5000, 301 N. King Street, Shepherdstown, WV, 25443-5000, USA.
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[†]Current address: Department of Biochemistry, Midwestern University, Science Hall 421-G, 555 31st Street, Downers Grove, IL, 60515, USA

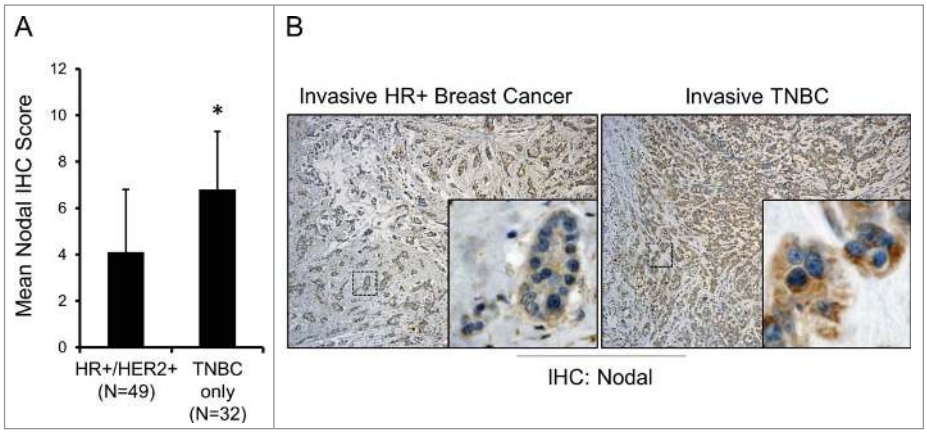

Figure 1. Nodal is highly expressed in triple-negative breast cancer (TNBC). (A) Results from immunohistochemistry (IHC) show mean Nodal scores for breast cancer with any hormone receptor (HR) or HER2 positive expression compared to TNBC. ($^{*}p = <0.05$, error bars represent standard deviation (SD)). (B) Representative images showing Nodal IHC staining in invasive HR positive breast cancer versus TNBC. (10X original magnification; inset: 63X magnification).

related to survival. These findings provide new insights into an important underlying advantage related to Nodal expression in TNBC, and potential rationale for combining anti-Nodal therapy with conventional approaches.

Results

We previously reported the detection of high levels of Nodal protein in TNBC ($n = 20$) compared to benign disease.[17] To advance these initial observations, we compared Nodal expression in TNBC to other invasive breast cancer subtypes using an additional 32 TNBC samples and 49 invasive breast cancer samples of differing HR and HER2 status. Scoring immunohistochemistry (IHC) samples for Nodal revealed significantly higher levels in TNBC compared to any HR/HER2 positive cases (Fig. 1A). Representative Nodal IHC for these findings is shown in Figure 1B. These data indicate that higher levels of Nodal are found within TNBC even when compared to invasive receptor positive tumors (summarized in Table 1), and are in agreement with studies that have reported correlations with Nodal expression and aggressive cancer-related characteristics.[13,15,18-23] Collectively, our results demonstrate the robust presence of Nodal in TNBC samples, representing a potential new target for this disease.

Chemotherapy is a mainstay of treatment for TNBC patients, but recurrence and lack of response to therapy represent significant challenges, especially in patients who develop metastatic disease. Based on our observations with clinical samples, we sought to evaluate the *in vitro* effects of Nodal inhibition in cells treated with doxorubicin. Doxorubicin is a potent topoisomerase II inhibitor and induces DNA damage through a variety of mechanisms.[24] While different treatment regimens for TNBC exist, doxorubicin has frequently been used alone, or in combination with other agents such as taxanes, cyclophosphamides and 5-flurouracil.[5,6,25,26] Thus, as an initial analysis, we examined the effects of doxorubicin on 3 TNBC cell lines: MDA-MB-231, BT549 and MDA-MB-468.[27] We sought to mimic a sequential therapy at concentrations which more closely approach achievable therapeutic plasma concentrations in patients.[28-30] We identified lower doses of doxorubicin in which cells recovered following cessation of treatment for 48hr. Cells treated with 5–10 nM doxorubicin exhibited minimal changes to overall viability and maintained proliferative capacity compared to higher doses (Fig. 2A, B). However, doxorubicin remained capable of inducing DNA damage despite these lower concentrations as demonstrated by an increase in phosphorylation of histone 2A.X at serine 139 (pH2A.X^{Ser139}), thus distinguishing these cells from non-treated controls and altering their genomic integrity (Fig. 3). Previous studies using anti-Nodal neutralizing antibodies have demonstrated changes to signaling pathways and molecular markers including reduced phosphorylation of histone 3 at serine 10 (pH3^{Ser10}), and a reduction in the pro-form of Nodal in cell lysates, which may be a result of inhibition of Nodal auto-regulatory mechanisms.[13,16,31] In doxorubicin treated cells, treatment with anti-Nodal antibody led to decreases in both Nodal protein levels and pH3^{Ser10}, demonstrating that Nodal expression is retained following exposure to doxorubicin and remains targetable (Fig. 4). Thus, following a 48hr recovery period, doxorubicin treated cells were then grown in the presence or absence of anti-Nodal antibody and functional effects were compared to cells treated with a rabbit polyclonal IgG isotype control,

Table 1. Nodal expression levels according to biomarker expression status in breast cancer.

Type of Cases	No. of cases (%)	Mean Nodal score	t-test (vs any HR/HER2)	t-test (vs TNBC)
All Breast Cancers	81 (100%)	5.2 +/− 2.9	*p* value	*p* value
ANY Hormone Receptor (HR) or HER2 status	49 (60%)	4.1 +/− 2.7	—	<0.001
HR positive/HER2 positive	14/49 (29%)	3.4 +/− 2.4	0.63	<0.001
HR positive/HER2 negative	27/49 (55%)	4.6 +/− 2.9	0.39	<0.01
HR negative/HER2 positive	4/49 (8%)	4.5 +/− 3.1	0.84	>0.23
HER2 unknown	4/49 (8%)	3.0 +/− 1.2	0.72	<0.001
Triple Negative Breast Cancer (TNBC)	32 (40%)	6.8 +/− 2.5	<0.001	—

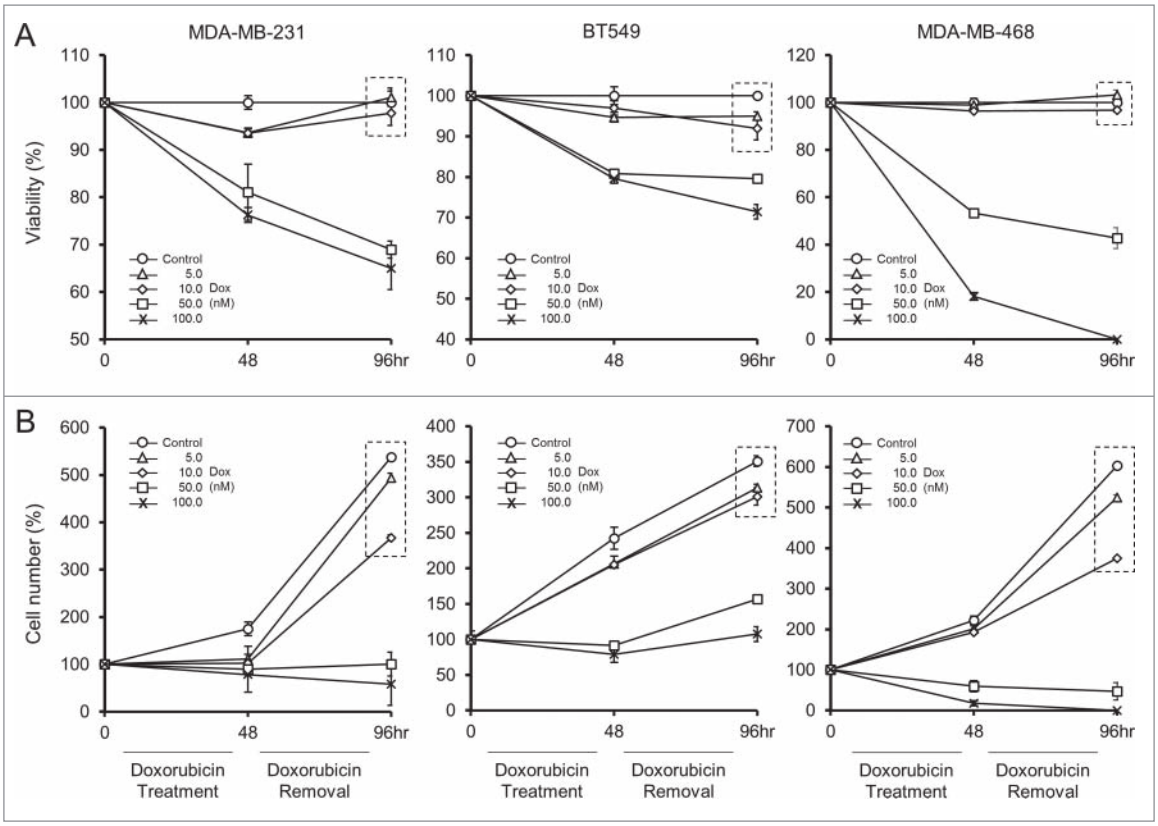

Figure 2. TNBC cell lines recover from therapeutic doses of doxorubicin. MDA-MB-231, BT549 and MDA-MB-468 TNBC cells were treated with increasing concentrations of doxorubicin for 48hr followed by removal of doxorubicin for an additional 48hr. Cells were analyzed by flow cytometry for overall viability (A) and proliferative capacity (B) at the end of treatment (48hr) and recovery (96hr). Data are normalized to percent viability of untreated cells (A) and cell number as a percentage of the initial population (B). (Error bars represent standard deviation; boxes represent concentrations used to assess subsequent Nodal inhibition).

as well as cells not exposed to doxorubicin – to evaluate if combinatorial effects could be achieved. Cell lines exhibited differences in their sensitivities to doxorubicin or anti-Nodal treatments alone; therefore, a range of concentrations of doxorubicin and anti-Nodal antibody were evaluated (from 1 to 10 nM doxorubicin with 2 to 4 μg/ml anti-Nodal antibody as shown in Fig. 5). In each cell line, concentrations were identified that exhibited improved effects on the reduction of viable cell populations and increased cell death when doxorubicin was followed by anti-Nodal treatment, which were greater than either treatment alone (Fig. 5A, B).

To explore these results further, and to determine the contribution of different stages of apoptosis underlying the observed cell death, levels of early and late apoptotic cells were assessed by flow cytometry. Inhibition of Nodal following doxorubicin caused an increase in early and/or late stage apoptosis when compared to doxorubicin or antibody treatments alone (Fig. 6A). To begin to provide potential molecular explanations for these effects, we questioned whether response pathways to DNA damage were altered in these cells. In cancer cells containing mutated p53, responses to DNA damage induced by doxorubicin are mediated primarily through the Chk1 and p38 stress response pathways, and p38 has been shown to promote survival of human cancer cells treated with doxorubicin by alteration of genes involved in apoptosis.[32-35] The MDA-MB-231, BT549 and MDA-MB-468 cell lines used in this study contain mutations within the *TP53* gene.[36-39] Thus, we examined these pathways in doxorubicin and anti-Nodal antibody treated cells. In anti-Nodal treated MDA-MB-231 cells, Nodal inhibition led to activation of the Chk1 DNA damage response as assessed by phosphorylation of Chk1 at serine 345 (pChk1^{Ser345}), indicating that Nodal alone may play a role in maintaining genomic integrity in these cancer cells (Fig. 6B). Strikingly, we also observed a dramatic decrease in the phosphorylation of p38$^{Thr180/Tyr182}$ in response to Nodal inhibition that was further reduced in the context of doxorubicin treatment (Fig. 6B). Collectively, these observations provide new clues into the survival pathways relatively unaffected by doxorubicin treatment alone, but notably counteracted by adding anti-Nodal therapy, resulting in mechanisms underlying DNA damage and cellular stress in TNBC – an important area of investigation for future studies (Fig. 6C).

Discussion

Lack of targetable molecules, together with relapse following chemotherapy, pose significant challenges to the treatment of TNBC. Novel targets and a better understanding of response to therapy remain important research areas for this disease, and represent opportunities for new therapeutic strategies. For example, recent findings that have reported the embryonic morphogen Nodal as a driver of cancer cell growth, plasticity, and highly expressed in aggressive cancers, are complemented by experimental studies showing Nodal inhibition resulting in suppression of tumorigenesis and the cancer stem cell phenotype.[13,17,31] In this study, we analyzed Nodal expression in a group of invasive breast cancer samples and found Nodal to be

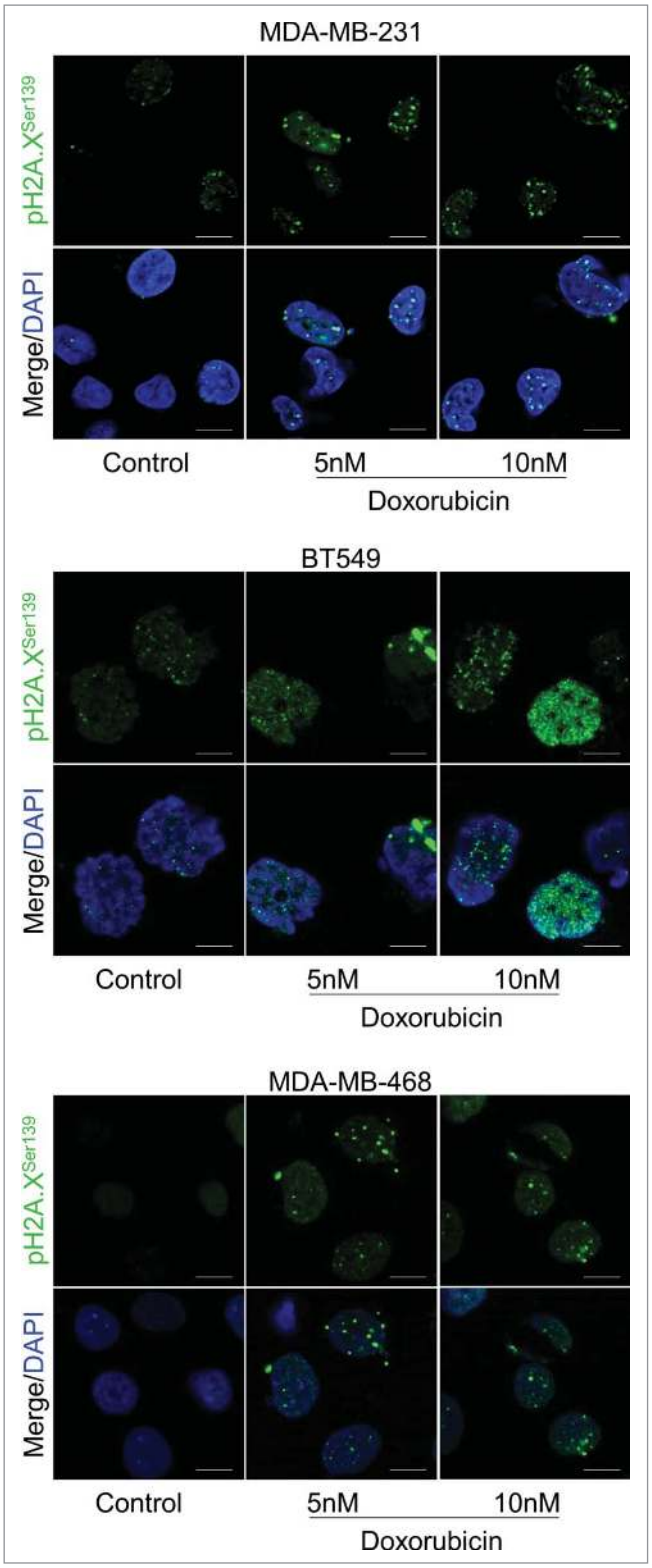

Figure 3. Doxorubicin treatment induces DNA damage. MDA-MB-231, BT549 and MDA-MB-468 cells were treated with indicated concentrations of doxorubicin for 48hr and allowed to recover for 48hr. Surviving cells were re-plated and grown for an additional 96hr. Cells were then fixed and analyzed for DNA damage by immunofluorescence of phosphorylated histone 2A.X at serine 139 (green). Cell nuclei stained with DAPI (blue). (Scale bar = 10 μm).

more highly expressed in TNBC when compared to invasive HR and HER2 positive breast cancers. These data advance a previous study in which Nodal expression was found to be significantly higher in TNBC compared to benign breast tissue.[17]

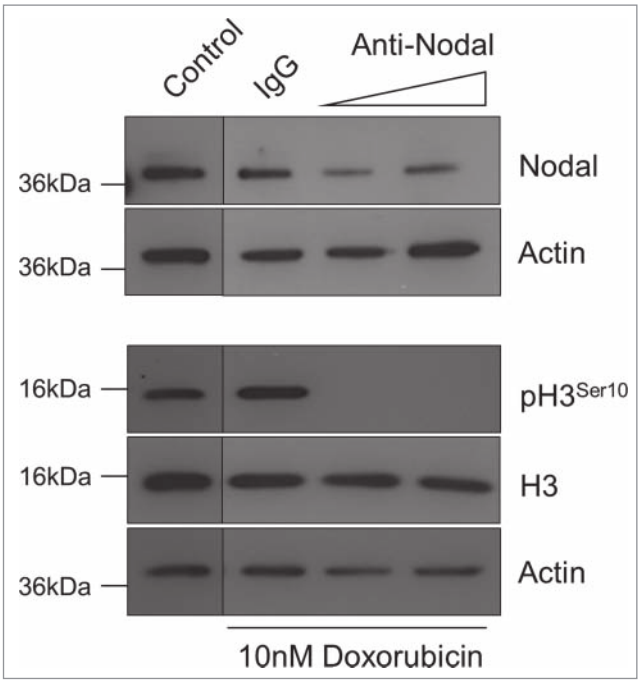

Figure 4. Nodal is a targetable molecule in doxorubicin treated TNBC cells. Immunoblot analysis of doxorubicin treated (10 nM) MDA-MB-231 cells in the presence of a neutralizing anti-Nodal antibody (2, 4 μg/ml) or IgG isotype control (4 μg/ml) for 96hr. Membranes were blotted for Nodal and phosphorylation of histone 3 at serine 10 (pH3^{Ser10}) compared to levels of total histone 3 (H3). Actin served as a loading control.

These results also support the prospect that Nodal represents a targetable molecule for TNBC and other receptor-negative breast cancers in which current targeted therapies are not effective. In line with this observation, a previous study identified Nodal as a prognostic marker for aggressive breast cancer independent of HR status or HER2 expression.[13]

Experimental inhibition of Nodal in combination with or subsequent to chemotherapy has decreased cancer cell growth and survival in multiple models. For instance, blocking Nodal signaling in pancreatic carcinoma in conjunction with gemcitabine led to reduced tumorigenesis *in vivo* and increased apoptosis of cancer cells.[15] Neutralizing Nodal in melanoma cells that survived treatment with the alkylating agent dacarbazine resulted in decreased cellular viability and invasion.[16] In support of these collective findings, we demonstrate that surviving populations of TNBC cell lines exposed to doxorubicin are sensitive to Nodal inhibition in the post-treatment period, exhibiting decreases in cellular growth and viability. A commonality among these studies is the use of agents that reduce the integrity of DNA within cancer cells. Notably, doxorubicin treated breast cancer cells receiving anti-Nodal antibody displayed differences in the activity of cellular stress (p38) and repair (Chk1) pathways, indicating that Nodal may play a role in response to cellular damage and represent an important factor in therapeutic approaches and patient overall response. This hypothesis is further supported by the stem cell-like phenotype which Nodal imparts to neoplastic cells, given the intrinsic ability of stem cells to survive cellular and genotoxic stress. Reduction of Nodal may impair basal DNA repair, heightening the effects of an agent such as doxorubicin. Since p38 induces pathways that act to resolve cellular stress such as MK2, reduction of this signaling by Nodal inhibition in the post-

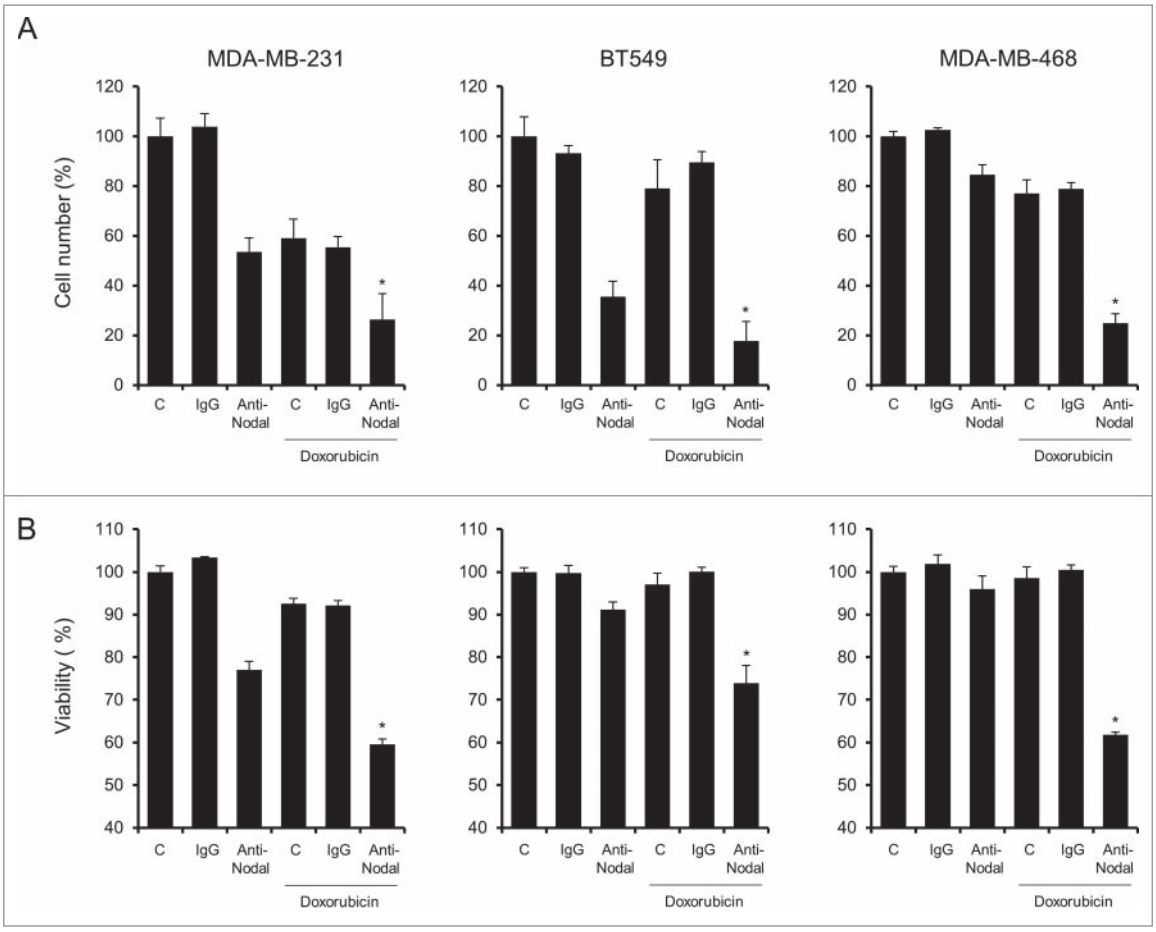

Figure 5. Inhibition of Nodal following doxorubicin treatment inhibits cancer cell growth and viability. Doxorubicin treated and untreated breast cancer cells were grown in the presence or absence of an anti-Nodal neutralizing antibody or isotype control IgG. Concentrations of doxorubicin and anti-Nodal antibody which exhibited combinatorial effects are shown (MDA-MB-231 and MDA-MB-468: 10 nM doxorubicin + 2 μg/ml anti-Nodal antibody; BT549: 1 nM doxorubicin + 4 μg/ml anti-Nodal antibody). Cells were analyzed by flow cytometry for viable cell number (A) and overall viability (B) compared to untreated and isotype control (4 μg/ml). Results are normalized to percent control conditions. ($^*p = <0.05$ when compared individually to all other treatment conditions; error bars represent standard deviation).

treatment phase may alter the ability of cells to appropriately respond to exposure to doxorubicin treatment.

In this brief report, we demonstrate high levels of Nodal expression in TNBC patient samples and highlight the anti-cancer effects of inhibiting Nodal in doxorubicin treated TNBC cells *in vitro*. These observations may be mediated through changes in DNA damage responses, and warrant further scrutiny to examine a potential relationship that could be leveraged between Nodal and current chemotherapies. However, Nodal signaling in cancer cells is mediated through canonical and non-canonical pathways that are not yet fully elucidated.[10] Combined with the complexities of DNA damage signaling and repair mechanisms, a careful delineation of these potential relationships will be required. Nevertheless, targeting Nodal in aggressive cancers remains an area of great interest and translational promise, especially as supportive evidence continues to emerge, and new clues are revealed relevant to critical signaling pathways underlying cellular survival.[31,40]

Materials and methods

Cell culture

MDA-MB-231 (HTB-26), BT549 (HTB-122) and MDA-MB-468 (HTB-132) TNBC cell lines were obtained from the American Type Culture Collection (ATCC) and grown in RPMI (Life Technologies, 31800–089) supplemented with 10% FBS and gentamycin. Cell lines were authenticated by short tandem repeat genotyping by PCR amplification at the Molecular Diagnostic/HLA Typing Core at Ann and Robert H. Lurie Children's Hospital of Chicago (2009–2010). Cell lines were used for less than 20 passages after thaw. All cultures were routinely screened for *Mycoplasma* (Roche, 111925910).

Chemicals and reagents

Doxorubicin (44583) was purchased from Sigma and diluted in sterile ultra-purified water (Milli-Q, Millipore). Working stocks were made at time of experiments. Rabbit polyclonal anti-Nodal antibody (sc-28913) was purchased from Santa Cruz Biotechnology and dialyzed to remove preservative. Antibodies for phospho-histone 3^{Ser10} (3377), histone 3 (9715), phospho-$Chk1^{Ser345}$ (2348), Chk1 (2360), phospho-$p38^{Thr180/Tyr182}$ (4511), p38 (8690) and phospho-histone $2A.X^{Ser139}$ (9718) were purchased from Cell Signaling Technologies. Anti-rabbit IgG Alexa Fluor 488 conjugate was purchased from ThermoFisher Scientific (A-11034). Anti-actin mouse monoclonal antibody was purchased from Millipore (MAB1501) and used to assess immunoblot loading.

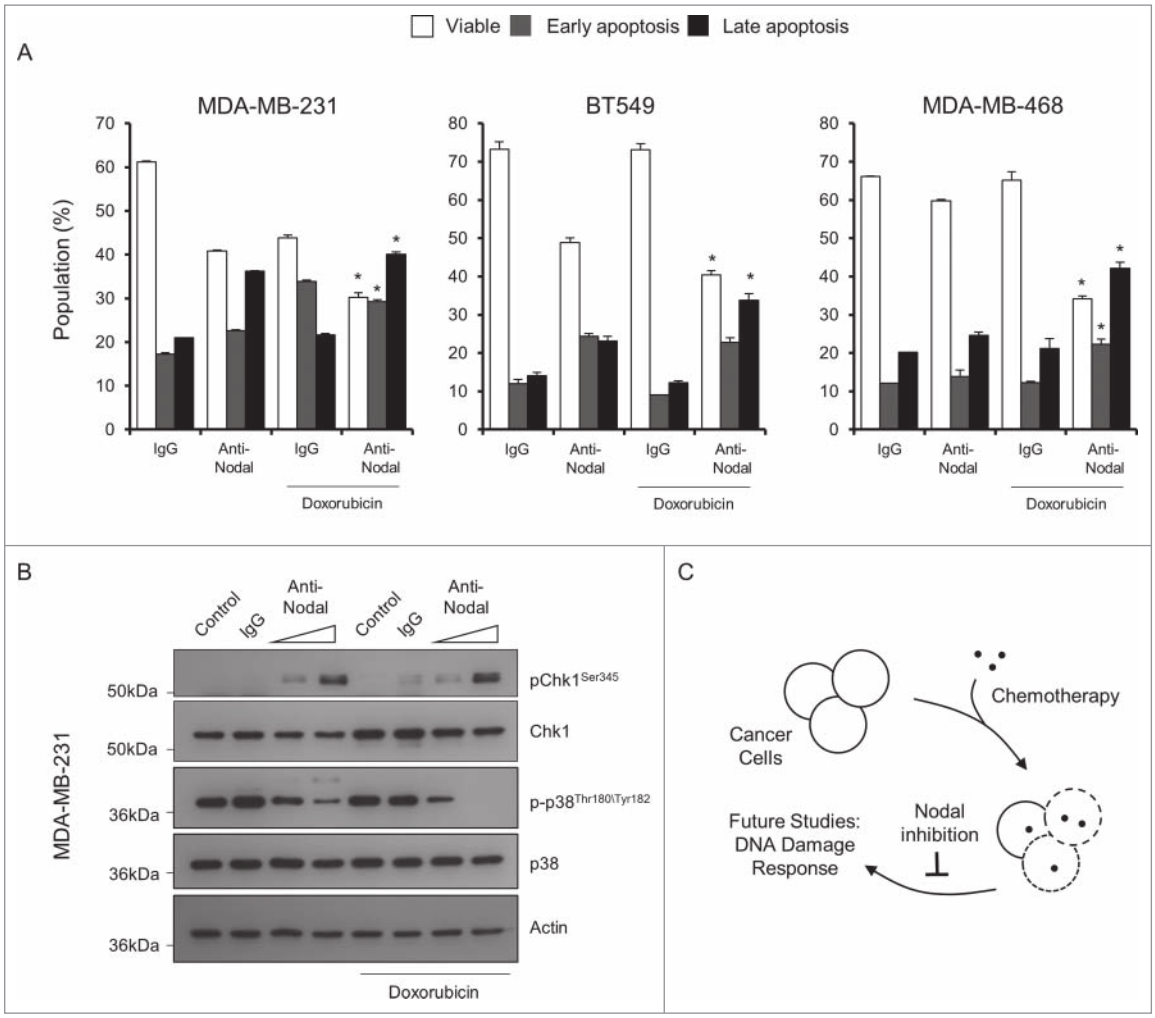

Figure 6. Inhibition of Nodal following doxorubicin treatment increases apoptosis and alters cellular stress pathways. (A) TNBC cells were treated as in Figure 5 and apoptotic populations were determined by flow cytometry using a DNA binding dye exclusion assay coupled with annexin V staining to designate early and late apoptosis. (* $p = <0.05$ when compared individually to all other treatment conditions; error bars represent standard deviation). (B) Immunoblot analysis of lysates probed for phosphorylation of Chk1 at serine 345 (pChk1^{Ser345}) and dual phosphorylation of p38 at Threonine 180 and Tyrosine 182 (p38$^{Thr180/Tyr182}$). Blots were probed for total Chk1 and p38 levels. Actin served as a loading control. (C) Summary of potential future studies examining the role of Nodal in response to cellular stress and DNA damage induced by chemotherapy.

Confocal fluorescence microscopy

Cells were grown on glass coverslips with indicated treatments and fixed in ice cold methanol, blocked in 2% BSA and incubated with primary and fluorescent secondary antibodies. Coverslips were mounted on glass slides using VectaShield with DAPI (Vector Laboratories, H-1200). Confocal images were obtained on a Zeiss 510 META Confocal Laser Scanning Microscope. Immunofluorescence data were analyzed using Zeiss ZEN2 Blue Windows®-based software.

Cell lysis and immunoblotting

Whole cell lysates were prepared in 25 mmol/L Tris pH 7.4, 0.5 mmol/L EDTA, 5% glycerol, 1% SDS with 1x Complete Mini protease inhibitors (Roche, 11836153001) and 1x PhosSTOP phosphatase inhibitors (Roche, 4906845001) with passage through a 21-gauge needle 12x on ice. Protein concentrations for lysates were determined by BCA assay (Thermo Scientific, PI23225) and diluted in Laemmli Sample Buffer (BioRad, 161–0737) supplemented with β-mercaptoethanol and boiled for 10 minutes at 95°C. 12% SDS-PAGE with 4% stacking gels were used to resolve lysates, with 10 μg of protein loaded per lane. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, 162–0184) and identified using the appropriate primary and secondary HRP-conjugated antibodies with chemiluminescence detection.

Flow cytometry

Flow cytometry was performed on a Guava easyCyte HT System Flow Cytometer (Millipore) using ViaCount reagent (Millipore, 4000–0040) for analysis of viability and cell number and Nexin (Millipore, 4000–0450) reagent for analysis of early and late apoptotic cells according to the manufacturer's instructions (Millipore). Doxorubicin treated cells were generated by treatment with indicated concentrations of doxorubicin for 48hr. Cells were then washed 3x with complete media and grown an additional 48hr to allow cells to recover. Surviving cells were re-plated at equal density with untreated controls

and grown in the presence or absence of anti-Nodal antibody or IgG isotype control for 72 - 96hr prior to analysis. Parameters were set using untreated cells. Representative data are shown with standard deviation. Student's t-test was used for statistical analysis.

Immunohistochemistry and patient samples

Breast cancer tissue samples were collected from a total of 81 patients with varying HR status and HER2 expression (Table 1). The tissue sections were processed for IHC as previously described.[13] Briefly, following antigen retrieval and blocking steps, sections were incubated in primary antibody for 60 min, followed by appropriate biotinylated secondary antibody (Biocare Medical, GM601H), and then streptavidin peroxidase (Thermo Scientific Lab Vision, TS125HR). Brown stain was developed with 3,3′-diaminobenzidine substrate (Thermo Scientific Lab Vision, TA125HDX) and sections were counterstained with hematoxylin (Biocare Medical, NM-HEM). As a negative control, adjacent serial sections were incubated with species appropriate irrelevant IgG (Jackson ImmunoResearch Labs, 015–000–003) at the same concentration as the primary antibody: mouse monoclonal anti-Nodal [Abcam, ab55676, 1:200]. The quality and intensity of staining were analyzed and scored at low power and high power in order to calculate an Index Score (IS), as previously described. Student's t-test was used for statistical analysis of the data. p value <0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

Mary J. C. Hendrix and Elisabeth A. Seftor hold a patent for targeting Nodal.

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