**ΔNp63α-Mediated Activation of Bone Morphogenetic Protein Signaling Governs Stem Cell Activity and Plasticity in Normal and Malignant Mammary Epithelial Cells**

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**Abstract**

Genetic analysis of TP63 indicates that ΔNp63 isoforms are required for preservation of regenerative stasis within diverse epithelial tissues. In squamous carcinomas, TP63 is commonly amplified, and ΔNp63α confers a potent survival advantage. Genome-wide occupancy studies show that ΔNp63 promotes bidirectional target gene regulation by binding more than 5,000 sites throughout the genome; however, the subset of targets mediating discreet activities of TP63 remains unclear. We report that ΔNp63α activates bone morphogenetic proteins (BMP) signaling by inducing the expression of BMP7. Immunohistochemical analysis indicates that hyperactivation of BMP signaling is common in human breast cancers, most notably in the basal molecular subtype, as well as in several mouse models of breast cancer. Suppression of BMP signaling in vitro with LDN193189, a small-molecule inhibitor of BMP type I receptor kinases, represses clonogenicity and diminishes the cancer stem cell population. Importantly, LDN193189 blocks reconstitution of mixed ALDH¹⁺/ALDH⁻ cultures indicating that BMP signaling may govern aspects of cellular plasticity within tumor hierarchies. These results show that BMP signaling enables reversion of committed populations to a stem-like state, potentially supporting progression and maintenance of tumorigenesis. Treatment of a mouse model of breast cancer with LDN193189 caused reduced expression of markers associated with epithelial-to-mesenchymal transition (EMT). Furthermore, in vivo limiting dilution assays revealed that LDN193189 treatment suppressed tumor-initiating capacity and increased tumor latency. These studies support a model in which ΔNp63α-mediated activation of BMP signaling governs epithelial cell plasticity, EMT, and tumorigenicity during breast cancer initiation and progression. Cancer Res; 73(2); 1020–30. ©2012 AACR.

**Introduction**

TP63 plays important roles in tumorigenesis, senescence, and epithelial stem cell regulation. It is rarely mutated in human cancers; however, genomic amplification of TP63 is observed in 10% of head and neck squamous cell carcinomas, 13% of serous ovarian carcinomas, 23% of squamous cervical carcinomas, and 28% of lung squamous cell carcinomas (¹). TP63⁻/⁻ mice exhibit developmental defects, including limb truncations, craniofacial abnormalities, and loss of stratified epithelia (², ³). Additional analysis indicates that ΔNp63 isoforms preserve long-term regenerative stasis in epithelial tissues (⁴). ΔNp63α is the predominant isofrom in normal and malignant breast tissue and is selectively expressed in the basal/myoepithelial cell layer that houses mammary stem cells (², ⁵, ⁶). In basal breast cancers and head and neck squamous cell carcinomas (HNSCC), ΔNp63α confers a survival advantage by actively repressing proapoptotic transcriptional programs (⁷, ⁸). In addition, ΔNp63α suppresses oncogene-induced senescence, suggesting it plays a role in cancer initiation (⁹, ¹⁰). These findings implicate ΔNp63α in cancer initiation and progression and suggest that this gene may be targeted for therapeutic benefit.

Bone morphogenetic proteins (BMP) are members of the TGFβ superfamily of cytokines that regulate diverse cellular processes, including cell fate specification, embryonic patterning, and maintenance of developmental potency in embryonic and adult stem cells (¹¹). BMPs bind to heterotetrameric complexes consisting of 2 type II receptors and 2 type I...
receptors. BMP-specific type II receptors (BMPRII, ActRII, and ActRIIB) possess constitutively active kinase domains that upon tetramerization, phosphorylate and activate BMP-specific type I receptor kinases (ALK2, ALK3, and ALK6). Activated type I receptors phosphorylate BMP-specific receptor SMADs (SMADs 1, 5, and 8). This promotes interaction with SMAD4 and subsequently causes nuclear translocation and target gene transcription (12). The pathway is negatively regulated by secreted antagonists such as Noggin and Gremlin and by inhibitory SMADs (SMAD6 and 7; ref. 12). Beyond its role in embryonic patterning, BMP signaling preserves prolonged replicative potential of adult stem cells by promoting cellular quiescence (13–15). In these studies, BMP signaling ablation caused increased proliferation of stem cell populations (13–15).

The role of BMP signaling in breast cancer initiation and progression remains unclear. Conflicting studies report that BMP signaling exerts either tumor-suppressive or tumor-promoting effects (16–22). The human genome encodes more than 20 BMP ligands and 10 BMP antagonists (23), suggesting that context-specific receptor–ligand combinations account for distinct cellular responses. Recent studies have focused on the effect of individual ligands or receptors on cellular processes; however, the comprehensive effect of BMP signaling on breast cancer progression remains unknown. LDN193189, a potent inhibitor of BMP type I receptor kinases, provides the opportunity to evaluate the effects of BMP signaling in diverse developmental, physiologic, and pathophysiologic processes. Here, we describe a regulatory relationship in which ΔNp63α activates BMP signaling via increased expression of BMP7 in the mammary epithelium. We show that BMP signaling is elevated in human breast tumors relative to matched normal tissue controls and in diverse mouse models of breast cancer. Suppression of BMP signaling with LDN193189 reduced clonogenicity and ALDH1 activity of mammary epithelial cells, indicating that LDN193189 inhibits the cancer stem cell phenotype (24, 25). Administration of LDN193189 to a murine breast cancer model decreased tumor incidence, tumor size, and expression of the epithelial-to-mesenchymal transition (EMT) marker vimentin. These studies support a model in which ΔNp63α-mediated induction of BMP7 contributes to the tumor stem cell phenotype. They also show that pharmacologic inhibition of BMP signaling may represent an opportunity to target cancer stem cells.

Materials and Methods

Cell culture and reagents

Breast cancer cell lines, MCF7, MDA-MB-231, SUM149, SUM102, SKBR3, BT20, HCC1937, and ZR75 were maintained according to American Type Culture Collection guidelines. The immortalized mammary epithelial cell (IMEC) line has been previously described (26). Mouse HC11 cells were grown in RPMI-1640 supplemented with 10% FBS, 10 ng/mL EGF, 5 μg/mL insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin. MMTV-Myc primary tumor cells were cultured in mammary epithelial cell growth medium (Lonza) without bovine pituitary extract (BPE).

LDN193189 was purchased from Stemgent Technologies and solubilized in dimethyl sulfoxide (DMSO). For in vivo studies, LDN193189 was synthesized by Aberjona Laboratories, Inc. and solubilized in sterile H2O for injection. Recombinant BMP7 (used at 50 ng/mL) and recombinant Noggin (used at 125 ng/mL) were purchased from R&D Systems. For neutralizing experiments, a rabbit polyclonal antibody to BMP7 was used at 500 ng/mL. (Abcam).

Western blot analysis

Cell lysates were prepared in NETN buffer (100 mmol/L Tris-Cl [pH 7.8], 1 mmol/L EDTA, 100 mmol/L NaCl, and 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche). Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were immunoblotted for p-SMAD1/5/8 (Cell Signaling), total SMAD1/5/8 (Cell Signaling), p63 (4A4 clone; Sigma), vimentin (Lab Vision), E-Cadherin (BD Biosciences), and β-actin (Cell Signaling) and visualized by enhanced chemiluminescence (Amersham Pharmacia).

Colony formation assay

Immortalized and transformed mammary epithelial cell lines were plated at 500 cells per well in 6-well plates. Cells were treated with the indicated concentrations of LDN193189 or vehicle control every 48 hours for 10 to 14 days. Cells were then fixed in methanol and stained with 0.1% crystal violet.

Quantitative real-time PCR analysis

RNA was isolated with the RNeasy Kit (Qiagen). cDNA was prepared using the iScript cDNA Synthesis Kit (BioRad). Quantitative PCR was carried out with iQ SYBR Green Super mix (BioRad) and oligonucleotide primers specific to each target gene. Relative changes in gene expression were obtained using the 2^–ΔΔCt method normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Animals

FVB wild-type (wt) and MMTV-Myc transgenic mice were maintained according to institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee at Dartmouth Medical School (Hanover, NH).

Human tissue samples

Formalin-fixed, paraffin-embedded (FFPE) human breast tumors and normal mammary tissue were obtained from the Tissue and Tumor Bank at Dartmouth Hitchcock Medical Center. The molecular subtype of human breast tumor samples was identified by the Department of Pathology at Dartmouth Hitchcock Medical Center.

Mouse tissue samples

Normal mammary glands from FVB wild-type and MMTV-Myc mice were fixed in Bouin solution for 24 hours. Tumor tissue from MMTV-Myc, MMTV-Wnt1, MMTV-HER2/Neu, MMTV-PyMT, and BRCA1 Δ1100/Δ1305; MMTV-Cre; p53−/− mice were fixed in 10% formalin for 24 hours. Fixed tissues were...
dehydrated through a series of graded alcohols and embedded in paraffin. Five-micrometer sections were applied to charged glass microscope slides.

**Immunohistochemistry**

Tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohol steps. Antigen retrieval was conducted by incubating sections in 10 μmol/L sodium citrate buffer for 20 minutes in a microwave oven. Peroxidase activity was quenched by incubating in 0.3% H2O2 for 30 minutes at room temperature. Sections were blocked for 2 hours in 5% goat serum/0.5% Tween-20/PBS at room temperature followed by incubation with p-SMAD1/5/8 primary antibody (1:100) diluted in blocking buffer at 4°C overnight in a humidified chamber. Sections were incubated with biotinylated secondary antibodies (Vector Laboratories) for 1 hour. Targets were detected with streptavidin-linked peroxidase (1:400, Vector Laboratories) for 30 minutes at room temperature and developed using the DAB Staining Kit and counterstained with hematoxylin.

**Immunofluorescence**

Following deparaffinization, rehydration, antigen retrieval, and blocking, sections were incubated in primary antibodies overnight at 4°C in a humidified chamber. Primary antibodies used were: p63 (1:100, 4A4 clone, Sigma), p-SMAD1/5/8 (1:100, Cell Signaling), and vimentin (1:100, Lab Vision). Sections were washed in PBS/0.1% Tween-20 and incubated in anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (1:500, Invitrogen). Sections were mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and imaged by fluorescence microscopy.

**Breast cancer allografts**

MMTV-Myc tumors were resected, and single-cell suspensions were prepared by incubation in collagenase/hyaluronidase for 6 hours at 37°C with periodic resuspension of the tissue. Cells were collected and incubated with trypsin-EDTA followed by Dispase and DNase I. Cells were then filtered through 40-μm cell strainers and resuspended in NH4Cl to lyse all red blood cells. For transplantation, cells were embedded in 100 μL of Matrigel and transplanted into the dorsal right flank of female FVB recipients. Mice were injected intraperitoneally with 2.5 mg/kg LDN193189 or vehicle control daily for 16 days and sacrificed 2 hours after the last injection.

**ALDEFLUOR assay**

Aldehyde dehydrogenase 1 (ALDH1) activity was assessed in IMECs, HC11s, and primary MMTV-Myc tumor cells using the ALDEFLUOR Kit (STEMCELL Technologies) per manufacturer’s protocol. Flow cytometric gates were based on diethylaminobenzaldehyde (DEAB)-treated negative controls. Samples were analyzed on a BD FACScan instrument using CellQuest software (BD Biosciences).

**Statistical methods**

Quantitative data are presented as mean values of triplicate points, and error bars represent the SEM.

**Results**

\( \Delta Np63 \alpha \) activates the BMP signaling pathway via enhanced expression of BMP7

Several lines of evidence support a role for BMP signaling in stem cell regulation where it maintains the proliferative potential of adult stem cells (27). Recent reports show an interaction between TP63 and BMP signaling. BMP7 expression is lost in TP63\(^{-/-}\) embryos (28), which is consistent with the finding that BMP7 is a transcriptional target of p53 family members, including \( \Delta Np63 \alpha \) (21). In addition, \( \Delta Np63 \alpha \) suppresses the inhibitory SMAD, SMAD7, in primary keratinocytes (29). To address the relevance of this relationship to breast cancer, expression levels of \( \Delta Np63 \alpha \) and BMP7 were measured by quantitative PCR in breast cancer cell lines. Results indicated a positive correlation between \( \Delta Np63 \alpha \) and BMP7 expression (Fig. 1A). Ectopic \( \Delta Np63 \alpha \) in human (IMECs) and mouse (HC11) immortalized mammary epithelial cell lines caused an 8- and 5.5-fold induction of BMP7 mRNA, respectively (Fig. 1B), indicating that \( \Delta Np63 \alpha \) was able to enhance BMP7 expression. Ectopic \( \Delta Np63 \alpha \) activates SMAD1/5/8 phosphorylation in a manner that is sensitive to either Noggin or BMP7-neutralizing antibodies (Fig. 1C). Ectopic TAp63\( \alpha \) also induced BMP signaling in IMECs (Supplementary Fig. S1A); however, \( \Delta Np63 \alpha \) expression is several thousand-fold greater than TAp63 expression in the mammary epithelium (Supplementary Fig. S1B). Treatment of IMECs with recombinant BMP7 rapidly and potently increased p-SMAD1/5/8. This effect could be inhibited by LDN193189, a selective inhibitor of BMPR1 kinase activity (Fig. 1D). LDN193189 also suppressed endogenous levels of BMP signaling in IMECs (compare lane 1 with lanes 4–6 in Fig. 1D). LDN193189 did not alter phosphorylation of TGFβ-specific SMADs (Fig. 1D), indicating specificity for BMP signaling over other TGFβ superfamily members. In addition, LDN193189 prevented \( \Delta Np63 \alpha \)-mediated phosphorylation of SMAD1/5/8 (Fig. 1E). Treatment of IMECs with recombinant BMP7 caused increased expression of the canonical target genes, Id1, Id2, and Id3 (Supplementary Fig. S2A). Ectopic \( \Delta Np63 \alpha \) enhanced expression of Id2 (Supplementary Fig. S2B), and this induction was suppressed by LDN193189, indicating that \( \Delta Np63 \alpha \)-mediated induction of Id2 partially depends on BMP type I receptor activity. These data support the conclusion that \( \Delta Np63 \alpha \) activates canonical BMP signaling via expression of BMP7 in the mammary epithelium.

**BMP signaling is elevated in human and mouse mammary tumors**

The relationship between \( \Delta Np63 \alpha \) and BMP signaling coupled to the role of \( \Delta Np63 \alpha \) in breast cancer initiation and progression suggested that BMP signaling might be hyperactivated in breast cancer. To address this, a tissue microarray containing breast cancer samples representing 5 molecular subtypes of breast cancer (30) was subjected to immunofluorescent analysis of p-SMAD1/5/8 expression. Results showed that BMP signaling was elevated in the majority of breast cancers relative to matched normal controls and was preferentially activated in basal breast tumors (Supplementary Fig. S3). Notably, 12 of 15 cases of the basal breast cancer subtype had levels of immunodetectable p-SMAD1/5/8 greater than the...
median expression level (Supplementary Fig. S3). To confirm this observation, 2-color immunofluorescence was used to determine BMP signaling status in normal human mammary epithelia and basal-subtype breast tumors. This analysis indicated that BMPR1A (Fig. 2A) and p-SMAD1/5/8 (Fig. 2B) levels are elevated in basal breast tumors relative to normal mammary gland tissue (Negative controls for immunohistochemical and immunofluorescent analysis can be found in Supplementary Fig. S4.) Robust levels of p-SMAD1/5/8 were also noted in tumors from MMTV-Myc, MMTV-PyMT, MMTV-Wnt-1, and MMTV-ErbB2/Neu mice relative to normal mammary gland controls (Fig. 2C). Interestingly, we noted that p-SMAD1/5/8 expression was widespread and not confined to specific cell types within the tumor tissue. These data indicate that BMP signaling is elevated in breast cancer and may be targeted for therapeutic benefit.

**Activation of BMP signaling is an early event in breast cancer**

There is an urgent need to identify pharmacologically accessible pathways that contribute to early stages of breast cancer. To address this, clinically defined samples of ductal carcinoma in situ (DCIS) were evaluated for levels of p-SMAD1/5/8. Co-staining with p63 revealed increased BMP signaling in luminal epithelial cells within DCIS lesions (Fig. 3A), suggesting that BMP activation may be an early event in breast cancer. This result also suggests a paracrine model in which ΔNp63α promotes BMP7 expression in basal epithelia resulting in activation of BMP signaling in neighboring luminal epithelia. To determine whether BMP signaling is hyperactivated in a premalignant state, p-SMAD1/5/8 levels were evaluated in 2 mouse models of breast cancer with distinct stages of premalignancy. In the MMTV-Myc model, parity shortens tumor latency and increases tumor penetrance to nearly 100%, suggesting that the parous MMTV-Myc mammary gland represents a model of premalignancy (31). Immunofluorescent analysis of p-SMAD1/5/8 in the mammary glands of unaffected nulliparous and parous MMTV-Myc mice revealed that BMP signaling is hyperactivated following parity and remains elevated through tumor progression (Fig. 3B). A second model of premalignancy in the mammary gland is the BRCA1<sup>Cov/Cov</sup>; MMTV-Cre; p53<sup>−/−</sup> model of basal breast cancer (32). Analysis of BMP signaling in this model showed robust levels of p-SMAD1/5/8 in unaffected mammary glands of tumor-bearing mice as well as in the tumor tissue, suggesting that BMP signaling is elevated before cancer initiation (Fig. 3C). Collectively, these studies indicate that hyperactivation of BMP signaling is an early event in tumorigenesis.
LDN193189 inhibits cancer stem cell activity in vitro

The established role for BMP signaling in preservation of replicative capacity and developmental potency of embryonic stem cells suggested a similar role in cancer stem cell regulation. To address this, we sought to determine whether disruption of BMP signaling affected aldehyde dehydrogenase 1-positive (ALDH1+) populations, which have been shown to be enriched for stem cells (25). Flow cytometric analysis of ALDH1 activity in IMECs and HC11 cells revealed that LDN193189 reduced the ALDH1+ populations in both cell lines (Fig. 4A and Supplementary Fig. S5A). Similar effects were observed in MMTV-Myc primary tumors cells where LDN193189 reduced the ALDH1+ population from 5.49% to 0.225% (Fig. 4B). These effects are dose-dependent and evident at doses at which LDN193189 is highly selective for BMP type I receptors (33). In addition, LDN193189 potently inhibits colony formation in IMECs (Fig. 4D) as well several immortalized and malignant mammary epithelial cells (Supplementary Fig. S5C and S5D). Conversely, treatment of IMECs with rhBMP7 increased clonogenic capacity (Supplementary Fig. S5B), suggesting that the anti-clonogenic effects of LDN193189 are due to disruption of BMP signaling. These data indicate that LDN193189 inhibits stem cell populations and clonogenic capacity in established mammary epithelial cell lines and primary murine tumor cells.

LDN193189 promotes mesenchymal-to-epithelial transition and restricts plasticity of mammary epithelial cells

EMT is an important cellular process that contributes to metastasis in response to ionizing radiation and chemotherapeutics (34), suggesting that EMT may represent an adaptive mechanism that enables cells to evade therapeutic assault. The ability of cells to undergo both EMT and the reverse process, mesenchymal-to-epithelial transition (MET), implies a state of cellular plasticity that may contribute to features of cancer stem cells. EMT is associated with decreased expression of epithelial cell adhesion proteins, notably E-cadherin and zonula occludens protein 1.
(ZO-1), and a corresponding increase in mesenchymal markers, such as vimentin and N-cadherin. It also renders cells more migratory, invasive, and resistant to apoptosis (34–36). The previous data in this study show that LDN193189 prevents colony formation. During these studies, we noted that LDN193189 causes IMECs to undergo morphologic changes that result in colonies that stain more intensely with crystal violet, indicating greater cell density and reduced migratory capacity (Fig. 5A). Staining of these colonies with ZO-1 revealed that LDN193189 treatment increases ZO-1 expression and promotes tight junction formation (Fig. 5A). Consistent with this observation, treatment of IMECs with LDN193189 for 72 hours led to increased expression of the epithelial marker E-cadherin and a corresponding decrease in expression of the mesenchymal marker, vimentin (Fig. 5B). These results implicate BMP signaling in the promotion of EMT in mammary epithelial cells. Recent reports have shown a direct link between induction of EMT and the acquisition of tumor initiating stem cell-like properties (37). Thus, this observation suggests that inhibition of BMP signaling opposes EMT and is consistent with the ability of LDN193189 to reduce the ALDH1⁺ stem cell-enriched population. To test whether LDN193189 is sufficient to restrict plasticity of epithelial cell populations, ALDH1⁺ and ALDH⁻/C0 fractions were cultured for 72 hours in the absence or presence of LDN193189. ALDH⁻ cells efficiently reconstituted the ALDH1⁺/ALDH⁻ parental culture and this effect was blocked by LDN193189 (Fig. 5C). The ability of the ALDH1⁺ fraction to reconstitute the ALDH1⁺/ALDH⁻ parental culture was accelerated in the presence of LDN193189, which is consistent with previous data showing that LDN193189 reduces the ALDH1⁺ population (Fig. 5C). These results indicate that BMP signaling confers plasticity and supports the cancer stem cell state (Fig. 5D).
LDN193189 exhibits in vivo activity in a syngeneic allograft model of MMTV-Myc–driven mammary tumorigenesis

To investigate the in vivo biologic activity of LDN193189, we established syngeneic allografts of MMTV-Myc driven breast cancer in 40 female FVB wild-type recipients. Two days post-transplantation, mice were randomized and injected intraperitoneally (i.p.) with 2.5 mg/kg LDN193189 or vehicle once daily for 16 days. Analysis of tumor lysates revealed that LDN193189 reduces p-SMAD1/5/8 levels and expression of DNp63a (Fig. 6A). The reduction in DNp63a is consistent with results showing that LDN193189 can inhibit the DNp63a-enriched ALDH1+ population. This effect was also observed in IMECs and HC11 cells treated with LDN193189 in vitro (Supplementary Fig. S6A and S6B). In addition, immunofluorescent analysis of these tumors showed that LDN193189 treatment decreased p-SMAD1/5/8 (shown in green) and vimentin (shown in red) expression, indicating that suppression of BMP signaling disrupts the EMT phenotype (Fig. 6B). Hematoxylin and eosin (H&E) staining of these tumors showed similar tumor morphology among both treatment groups (Supplementary Fig. S6).

LDN193189 reduces tumor-initiating ability and increases tumor latency

To assess the effect of LDN193189 on tumor-initiating capacity, limiting dilution transplants of cells pretreated with LDN193189 was conducted. Primary MMTV-Myc tumor cells were treated ex vivo with LDN193189 for 48 hours, sharply diminishing the ALDH1+ population compared with vehicle control (Fig. 7A). Treated and untreated cells were embedded in Matrigel and transplanted at limiting dilutions into female...
FVB recipients. Recipients of LDN193189-treated cells were treated with 2.5 mg/kg LDN193189 daily for 24 days. These mice displayed a marked decrease in tumor incidence and increased tumor latency (Fig. 7B). Tumor incidence results (Supplementary Fig. S7B) highlight the increased latency to progression among the LDN193189-treated animals. None of the mice transplanted with the fewest cells (10 cells) and treated with LDN193189 formed tumors, whereas 75% of mice receiving vehicle treatment at this cell dose formed palpable tumors (Fig. 7B, left). At the intermediate cell dilution (100 cells), 100% of the mice in the vehicle treatment group developed palpable tumors versus 50% of mice in the LDN193189 treatment group (Fig. 7B). Tumors were measured at the time of harvest, and while no group reached statistical significance, LDN193189-treated animals showed a decreased trend in tumor mass for all 3 cell doses tested (Supplementary Fig. S7B). The lack of statistical significance is likely attributed to the high variability in tumor mass even within each treatment group. Individual tumor mass data are shown in Supplementary Fig. S7C. Taken together, these data implicate that BMP signaling may be central to regulating epithelial cell plasticity, EMT, and stemness during breast cancer initiation.

Discussion

Breast cancer is the leading cause of cancer-related death in women worldwide, with incidence rates increasing globally over the last 25 years (38). In the United States, the lifetime risk of breast cancer for all women is 12.7% (38). Targeted molecular therapies exhibit less toxicity and enhanced tumor selectivity than broad-spectrum chemotherapeutics and have become important components of current breast cancer treatment modalities. Breast cancer progression and recurrence remain a formidable clinical challenge due to acquired resistance to existing therapies, inability to respond to adjuvant therapies, and a failure of drug combinations to produce durable remissions. This highlights the need for the identification of additional genetic alterations that underlie disease recurrence and subsequent design of selective inhibitors of...
these pathways for use in novel combination with conventional cytotoxic agents.

Studies presented here evaluate BMP signaling status by measuring the phosphorylation of SMAD1/5/8, which represents a point of convergence for all BMP signaling. We present evidence that BMP signaling is hyperactivated in human and mouse breast tumors relative to normal tissue controls and that it is highest in the basal breast cancer subtype. In addition, we present data indicating that activation of BMP signaling is evident in DCIS and in mouse models of premalignancy. Furthermore, numerous reports show a role for BMP signaling in promoting tumor cell migration and invasion (17, 19–21, 39–41), suggesting that BMP signaling may be targeted for therapeutic benefit. In vitro analysis of LDN193189, a small-molecule inhibitor of BMPR1 kinases, revealed that suppressing BMP signaling reduced the stem cell–enriched ALDH1+ population. Here, we show that ΔNp63α can induce BMP7 expression and activate canonical BMP signaling, providing a potential mechanism by which ΔNp63α preserves stem cell proliferative potential. Together, our data support the conclusion that BMP signaling may be targeted to reduce the activity of breast cancer stem cells.

In vivo studies revealed that biologically active doses of LDN193189 caused reductions in tumor size and volume in mice treated daily with LDN193189. These effects, however, did not reach statistical significance, which we attribute to the very rapid expansion of tumors in the syngeneic allograft model. Longer treatment windows in a model with growth rates that more closely approximate human breast cancers may yield statistically significant effects on tumor volume. An alternative explanation may be that LDN193189 is targeting the rare cancer stem cell populations within the tumor causing only modest changes in tumor volume. Administration of LDN193189 with an agent that targets the bulk tumor population may reduce tumor volume and the likelihood of recurrence and metastatic disease.

We observed decreased vimentin expression in tumor tissue from mice treated with LDN193189, suggesting that BMP inhibition causes these tumors to undergo MET. This is consistent with the pro-MET effects we observed with drug treatment in vitro. Limiting dilution analysis showed that LDN193189 restricts the tumorigenic capacity of MMTV-Myc allografts. Mice that do form tumors in the presence of LDN193189 administration exhibit an increased latency to palpation. These experiments show the ability of LDN193189 to repress breast tumor–initiating populations. These initial studies are promising, but much work is needed to understand the efficacy of this drug in cancer treatment and the toxicity to normal stem cell compartments.

Reactivation of normal developmental pathways is a common feature of cancer progression and has been observed previously with the Hedgehog and Wnt signaling pathways (42). Aberrant activation of these pathways during tumorigenesis is believed to lead to inappropriate specification of cells to a stem-like state. In this study, we show that BMP signaling is hyperactivated during breast cancer initiation and progression and that it enhances tumor stem cell populations and EMT, indicating a role for BMP in the pathophysiology of cancer. Overall, these data support a model in which BMP signaling, driven by ΔNp63, governs key...

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Table: Limiting dilution analysis reveals that LDN193189 reduces tumor-initiating ability and increases tumor latency. A, primary MMTV-Myc tumor cells were cultured ex vivo in the presence of absence of 1 μmol/L LDN193189 for 48 hours and subjected to fluorescence-activated cell sorting (FACS)-based analysis of ALDH1 activity. Cells were incubated with DEAB, an ALDH1 inhibitor, and used as a negative control. Gates were set according to the negative control. The cells were subsequently transplanted by limiting dilution into the dorsal right flanks of FVB wild-type female recipient mice. Mice were treated 2 days after transplantation daily for 24 days with 2.5 mg/kg LDN193189 or vehicle control by i.p. injection. B, tumor latency is plotted as time to palpation for each group of mice. n = 4 for each group.

Figure 7. Limiting dilution analysis reveals that LDN193189 reduces tumor-initiating ability and increases tumor latency. A, primary MMTV-Myc tumor cells were cultured ex vivo in the presence of absence of 1 μmol/L LDN193189 for 48 hours and subjected to fluorescence-activated cell sorting (FACS)-based analysis of ALDH1 activity. Cells were incubated with DEAB, an ALDH1 inhibitor, and used as a negative control. Gates were set according to the negative control. The cells were subsequently transplanted by limiting dilution into the dorsal right flanks of FVB wild-type female recipient mice. Mice were treated 2 days after transplantation daily for 24 days with 2.5 mg/kg LDN193189 or vehicle control by i.p. injection. B, tumor latency is plotted as time to palpation for each group of mice. n = 4 for each group.

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No potential conflicts of interest were disclosed.

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Malignant Mammary Epithelial Cells

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