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Emerging Roles of Nodal and Cripto-1: From Embryogenesis to Breast Cancer Progression

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Abstract

Breast carcinoma cells and embryonic progenitors similarly implement stem cell-associated signaling pathways to sustain continued growth and plasticity. Indeed, recent studies have implicated signaling pathways, including those associated with the Notch, and Transforming Growth Factor-Beta (TGF- β) superfamilies, as instrumental to both embryological development and breast cancer progression. In particular, Nodal, an embryonic morphogen belonging to the TGF- β superfamily, and its co-receptor, Cripto-1, are requisite to both embryogenesis and mammary gland maturation. Moreover, these developmental proteins have been shown to promote breast cancer progression. Here, we review the role of Nodal and its co-receptor Cripto-1 during development and we describe how this signaling pathway may be involved in breast cancer tumorigenesis. Moreover, we emphasize the potential utility of this signaling pathway as a novel target for the treatment and diagnosis of breast cancer.

Keywords

Nodal; Cripto-1; embryogenesis; mammary development; breast cancer

INTRODUCTION

Numerous genes have been shown to be expressed in embryonic stem cells and considered important during early embryonic development. These include, but are not limited to, Notch and its associated signaling transcription factors, such as HES [1,2], the GATA family of transcription factors [3]; the developmental pluripotency associated-3 (DPPA3) gene also known as STELLA [4]; stem cell related transcription factors like the POU domain transcription factor Oct4 [5]; members of the homeobox gene family, such as HOX [6] and

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zinc-finger transcription factors like snail and twist [7, 8]. Members of the Transforming Growth Factor- β (TGF- β) superfamily, including Nodal [9], Activin and GDF-1 and -3 [10] have also been documented as potent mediators of cell fate in both embryological and adult systems. Furthermore, changes in expression of some of these and other genes (Fig. 1) have been suggested to play a role in the formation of tumors such as breast cancer [11,12].

The establishment of human embryonic stem cell (hESC) lines has facilitated the identification of other stem cell-related genes. A recent study of almost 60 hES cell lines from several different laboratories worldwide has established the expression of associated genes illuminating a more complex molecular basis for pluripotency [13]. These included Nanog, Oct4, Nodal and Nodal's co-receptor teratocarcinoma-derived growth factor-1 (TDGF-1), also referred to as Cripto-1. Together with Nanog and Oct4, Nodal and Cripto-1 have been suggested to play a role in self-renewal and maintenance of pluripotency and are considered by some as markers for hESCs [14]. For example, Cripto-1 has been shown to be a direct target gene in embryonic stem (ES) cells for Nanog and Oct4 suggesting that many of the functions of Nanog and/or Oct4 may be mediated in part through expression of Cripto-1 [15]. In addition, Nodal maintains the pluripotency of ES cells and is one of the first genes to be down-regulated as totipotent ES cells differentiate during embryoid body formation. Moreover, inhibition of the Nodal signaling pathway, through pharmacological inhibition of its receptor, results in hESC differentiation [16]. This review will highlight the potential role of Nodal and its co-receptor Cripto-1 during mammary gland development and how this signaling pathway may be involved in breast cancer tumorigenesis and progression.

BIOCHEMICAL AND SIGNALING CHARACTERISTICS OF NODAL AND CRIPTO-1

The Nodal signaling pathway is tightly regulated by a myriad of transcriptional regulators, post-translational modifications and extracellular factors. The human Nodal gene, containing 3 exons, is located on Chromosome 10q22.1. In mice, Nodal expression is enhanced by at least 3 separate transcriptional regulatory regions, the Node Specific Enhancer (NDE), approximately 10 kb upstream of the gene locus, the Left Side specific Enhancer (LSE), approximately 4 kb upstream of the translational start site; and the ASymmetric Enhancer (ASE), located in the first intron [17–19]. Studies have determined that the LSE and the ASE are regulated in the mouse by Nodal via a positive feedback loop that culminates in the activation of FoxH1. In contrast, the NDE has been shown to induce Nodal expression in the mouse in response to Notch signaling through CBF1 binding elements in the promoter region [20,21]. Gene alignments indicate that the human Nodal locus contains similar enhancer elements, so it is likely that human Nodal expression is regulated in a similar manner. Indeed, our preliminary studies indicate that like mouse Nodal, human Nodal is up-regulated by Notch4 signaling in melanoma cells [22]. Moreover, a positive feedback loop, similar to that described for the LSE and ASE in mice, has been documented to sustain Nodal expression in human ES and melanoma cell types [23, 24]. As a complement to these canonical regulators of transcription, Nodal expression may also be governed by gene methylation and miRNA-directed degradation. For example, we have determined that there is a sizable CpG island (>1300 base pairs) near the transcription start site (TSS) of the *Nodal* gene, and this site may regulate Nodal expression [22]. Moreover, a novel miRNA (miR-430) has been shown to block the translation of a Nodal homolog, *squint*, in zebrafish [25]. MiR-430 target sites are also present in the mammalian *Nodal* gene; and so it is likely that Nodal expression is similarly affected by miRNA-mediated degradation in humans [26].

Nodal is also regulated post-translationally by subtilisin-like proprotein convertases, including PACE-4 and Furin [27], and by glycosylation. In a manner similar to most TGF- β family members, Nodal is synthesized as a pro-protein that is activated following proteolytic

processing by co-receptors at R-X-(K/R)-R and R-X-X-R consensus sequences [26]. Removal of the pro domain reduces Nodal stability and signaling range, thereby promoting autocrine signaling [28]. Conversely, glycosylation of mature Nodal provides the protein with increased stability so that it can potentiate paracrine signaling events [28]. Hence, post-translational modifications of Nodal are important mediators of Nodal signaling outcomes.

Nodal propagates its signal by binding to heterodimeric complexes between type I (ALK 4/7) and type II (ActRIIB) activin-like kinase receptors. Assembly of this complex, which often includes Cripto-1, results in the phosphorylation and activation of ALK 4/7 by ActRIIB, followed by the ALK 4/7 mediated phosphorylation of Smad-2 and possibly Smad-3 (Fig. 2). Phosphorylated Smad 2/3 subsequently associates with Smad-4 and then translocates to the nucleus where it regulates gene expression through an association with transcription factors such as FoxH1 and Mixer [26]. Nodal up-regulates its own transcription via a positive feedback loop. In order to regulate the levels of this potent morphogen, embryological systems employ Nodal inhibitors such as Lefty A, Lefty B, Cerberus and Tomoregulin-1 [26,29]. Extracellular Nodal inhibitors control Nodal signaling by spatially and temporally restricting the Nodal-mediated activation of ALK 4/7. For example, Lefty-A and B, highly divergent members of the TGF β superfamily, specifically antagonize the Nodal signaling pathway by binding to and interacting with Nodal and/or with Cripto-1 in a manner that blocks ALK activation [26,30]. This restriction of Nodal signaling can occur in the extracellular microenvironment, where Nodal and Cripto-1 are present, as well as at the cell surface. Of note, the Lefty proteins have not been found to bind ALK4 or ActRIIB; hence these Lefty proteins are not competitive inhibitors of the ALK receptor complex. Furthermore, in embryological systems, the Lefty genes are often downstream targets of Nodal signaling, thereby providing a powerful negative-feedback loop for this pathway [26,30].

Cripto-1 is a member of the EGF-CFC protein family [31]. EGF-CFC family members contain an NH₂-terminal signal peptide, a modified EGF-like region, a conserved cysteine-rich domain (CFC motif) and a short hydrophobic COOH-terminus that contains additional sequences for glycosyl-phosphatidylinositol (GPI) cleavage and attachment [32] (Fig. 3). Full activity requires the presence of a peptide containing only an intact EGF domain and CFC domain [33,34]. All EGF-CFC proteins contain a consensus O-linked fucosylation site within the EGF-like motif that was considered necessary for their ability to function as co-receptors for the TGF β -related proteins, Nodal or Vg1/GDF-1 [34, 35]. However, a recent report shows that it is not fucose which is necessary for the coreceptor function but rather the amino acid threonine to which the fucose is attached [36]. Although Cripto-1 can be found both as a cell-associated form (cis) and extracellular soluble form (trans), new findings have demonstrated that the GPI-attachment of Cripto-1 is required for the paracrine activity as a Nodal co-receptor [37].

During embryogenesis, EGF-CFC proteins function, as mentioned previously, as cell-surface co-receptors for Nodal through activation of the serine threonine kinase type I (ALK4) and type II (ActRIIB) receptor complex in *Xenopus* [26] (Fig. 2). Biochemical evidence has demonstrated a direct interaction between mouse Cripto-1 and ALK4 in *Xenopus* oocytes and this interaction is necessary for Nodal to bind to the ALK4/ActRIIB receptor complex and to stimulate Smad-2 phosphorylation and activation [38,39]. Cripto-1 can also directly interact with serine threonine kinase receptor ALK7 enhancing the ability of ALK7 to respond to Nodal [40]. These phenomena are perhaps best exemplified in Cripto-1 null mice which die at day 7.5 due to their inability to gastrulate [41,42]. Studies have determined that Nodal may also signal in a Cripto-1-independent fashion. For example, Reissmann and colleagues revealed that Nodal can bind to activate ALK 7 in the absence of Cripto-1, but that Cripto-1 markedly enhances this process [40]. Another study showed that

the Nodal precursor can bind to ALK 4 in the extraembryonic ectoderm of the developing mouse embryo in a Cripto-1-independent manner, and that this binding results in the expression of Nodal-responsive genes [43]. Finally, using murine knock out models, Liguori and colleagues recently demonstrated that Nodal can signal extensively and control axis specification in the absence of Cripto, if its inhibitor Cerberus is also inhibited [44]. More recent evidence also supports a role for Cripto-1 during anterior-posterior axis specification independently of canonical Nodal signaling pathway [45].

In addition to functioning as a coreceptor for Nodal, Cripto-1 has been shown to mediate signaling of other TGF- β ligands, such as Activin and Xenopus Vg1 and its ortholog in mouse GDF1 [46]. Similar to Nodal, Vg1 and GDF1 can bind to ALK4/ActRIIB receptor complex only in the presence of EGF-CFC co-receptors. In contrast, binding of Cripto-1 to Activin and TGF- β 1 can inhibit Activin and TGF- β 1 signaling in mammalian cells [47,48]. Cripto-1 can also activate the ras/raf/MAPK and PI3-K/AKT/GSK-3 β signaling pathways [49,50]. Activation of these two intracellular pathways is independent of Nodal and ALK4, since Cripto-1 can stimulate MAPK and AKT phosphorylation in EpH4 mouse mammary epithelial cells and MC3T3-C1 osteoblast cells that lack ALK4 and Nodal expression, respectively [39]. Activation of these signaling pathways can be achieved with a soluble GPI-truncated Cripto-1 recombinant protein and can occur through direct binding of Cripto-1 to Glypican-1, a membrane-associated heparan sulfate proteoglycan [49,51]. Binding of Cripto-1 to Glypican-1 activates MAPK and AKT signaling pathways via phosphorylation of the cytoplasmic tyrosine kinase c-Src [51] (Fig. 2).

The interactions between integrins, extracellular matrix and signaling molecules like fibronectin and FAK, respectively, are capable of regulating cellular processes like growth and development and have been shown to be involved in tumor proliferation, thus representing attractive targets for potential cancer therapy [52–55]. Overexpression of Cripto-1 *in vitro* and *in vivo* has been associated with increased expression of fibronectin and various integrins and with increased activation of focal adhesion kinase [56]. Moreover, Cripto-1 was found to bind to β -4 integrin in an immunoprecipitation assay (unpublished data). These data suggest that Cripto-1 may be involved in regulating integrin signaling either directly by binding to integrins and subsequently activating integrin signaling or indirectly by regulating the expression of extracellular matrix proteins which are also capable of binding integrins and activating integrin signaling (Fig. 2).

FUNCTION AND EXPRESSION OF CRIPTO-1 AND NODAL DURING EMBRYONIC DEVELOPMENT

Cripto-1 and Nodal converge during embryogenesis to regulate axis formation, mesendoderm induction, axial patterning, and left-right asymmetry. This signaling axis also plays a role in the maintenance of pluripotency in the epiblast and trophectoderm compartments [27, 57]. During early mouse embryogenesis, Nodal exhibits a dynamic expression pattern that lasts until mid gestation. Nodal mRNA is first detected at approximately 5 days post coitus (dpc) in the visceral endoderm and throughout the epiblast. At about the time of gastrulation, Nodal is restricted to the proximal posterior regions of the embryonic ectoderm and the primitive endoderm overlying the primitive streak. Shortly after, Nodal becomes intensely localized to the periphery of the node in the anterior primitive streak [58,59]. This node is equivalent to the dorsal lip, or Spemann's organizer in *Xenopus*, which initiates embryonic axis formation [26,60,61]. During somitogenesis, Nodal expression is increasingly restricted to the Left side of the Node, and by the 3–4 somite stage (8 dpc), Nodal is confined to a subpopulation of mesoderm cells on what will become the left side of the embryo [59]. This asymmetric Nodal expression precedes heart looping and

axial rotation, and persists to the 12–14 somite stage (8–9.25 dpc), after which Nodal expression is not detected [59,62].

Nodal plays an essential role in embryogenesis by acting as an organizing signal before gastrulation, to initiate axis formation [26,60,63]. Studies in zebrafish and mice have demonstrated that Nodal is required for mesoderm and endoderm formation. In mice, *nodal*-null embryos fail to form a primitive streak and are deficient in endoderm and mesoderm. Moreover, these mice die shortly after gastrulation. Zebrafish similarly require Nodal-like proteins (*cyclops* and *squint*) for mesoderm and endoderm induction. Indeed double mutants for *squint* (*sqt*) and *cyclops* (*cyc*) lack head and trunk mesoderm and fail to form a germ ring, which is similar to the primitive streak [58,62,64,65]. In addition, Nodal has been shown to induce secondary axis formation when ectopically engrafted into zebrafish blastulas [66]. Studies in zebrafish have also determined that Nodal specifies anterior-posterior patterning such that specification of the anterior axial mesoderm is induced by higher Nodal levels than are required for more posterior fates [67].

During early mouse embryogenesis, Cripto-1 mRNA expression is found in the embryonic ectoderm following implantation of the blastocyst. On day 6.5 of gestation, Cripto-1 is detected at increasing levels in the epiblast cells undergoing epithelial to mesenchymal transition (EMT) as they migrate through the nascent primitive streak and in the developing mesoderm cells [42, 68–70]. By day 7, Cripto-1 is detected mostly in the truncus arteriosus of the developing heart. With the exception of the developing heart, little if any expression of Cripto-1 mRNA can be detected in the remainder of the embryo after day 8 [32,68]. As previously mentioned, Cripto-1 null mice (Cripto-1^{-/-}) succumb at day 7.5 due to their inability to gastrulate and form appropriate germ layers [42]. Fibroblasts that were derived from Cripto-1^{-/-} embryos were impaired in their ability to migrate towards either fibronectin or type 1 collagen as compared to embryonic fibroblasts from wild type embryos [70]. In zebrafish, the Cripto-1 ortholog, *one-eyed pinhead* (*oep*), with the two Nodal-related genes *sqt* and *cyc*, is necessary for initiating mesoderm, endoderm and A/P axis formation [9,71, 72]. Mutations in *oep* result in cyclopia, absence of head and trunk mesoderm, loss of prechordal plate and ventral neuroectoderm, impairment of gastrulation movements, loss of A/P axis patterning and positioning and L/R laterality defects [71,72]. Rescue of the *oep* mutant phenotype can be achieved by expression of either full-length or secreted COOH-terminal truncated forms of the *oep* protein suggesting that *oep* can function under certain conditions as a paracrine effector. Ectopic expression of *Xenopus* FRL-1 or mouse Cripto-1 or overexpression of Activin or activation of downstream components in an Activin-like signaling pathway such as the ALK4 receptor (TARAM-A) or Smad-2 can also rescue *oep* inactivating mutations [72]. *Oep*, like mouse Cripto-1, is absolutely required for the migration of mesendoderm cells through the primitive streak [73].

The Nodal/cryptic signaling pathway is also involved in the establishment of the L/R embryonic axis, demonstrating that the same signaling molecules are utilized in multiple developmental pathways [9]. However, at this developmental stage with the exception of *oep*, cryptic replaces the function of Cripto-1 as the co-receptor for Nodal. In mice, Nodal is expressed symmetrically before and during gastrulation but is restricted to the left side of the node and lateral plate mesoderm during early segmentation. Nodal proteins are similarly localized to the left side in the chick, frog and zebrafish. This restriction is essential for L/R asymmetry as ectopic Nodal expression can cause defects in L/R asymmetry of the heart and gut in chick, *Xenopus* and zebrafish [62]. Moreover, in mice hypomorphic mutants lacking *nodal* expression in the left lateral plate, mesoderm exhibit defects in left-right body patterning resulting in organ anomalies including random heart looping [62].

Nodal signaling in zebrafish during L/R asymmetry development also requires a functional *oep* gene [61, 74,75]. Mutation and partial rescue of *oep* in the zebrafish or targeted disruption of cryptic in mice (*Cfc1*) and humans (*CFC1*) results in laterality defects including atrial-ventricular septal defects, pulmonary right isomerization, inverted positioning of abdominal organs (*situs inversus*) and holoprosencephaly [76,77]. Germline deletion of cryptic eventually leads to post-natal death at approximately 2 weeks because of severe cardiac malfunction [61,75].

CRIPTO-1 AND NODAL DURING MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

Cripto-1 mRNA can be detected in 4 to 12 week old virgin, mid-pregnant and lactating FVB/N mouse mammary glands [78,79]. Biologically active Cripto-1 protein has also been detected as a secretory component in human milk suggesting Cripto-1 secretion may be involved in regulating proliferation and differentiation of milk producing cells [80]. Further support for Cripto-1 regulation of mammary epithelial function comes from data showing decreased ability of mouse mammary epithelial cells to respond to the lactogenic hormones, dexamethasone, insulin and prolactin (DIP) when pre-treated with Cripto-1 and inhibition of β -casein expression, via p21*ras*- and phosphatidylinositol 3'-kinase (PI3K)-dependent pathways, when these cells were simultaneously treated with both Cripto-1 and DIP [81].

Transgenic mice models that overexpress the human Cripto-1 transgene (CR-1) have given some insight into the possible role of Cripto-1 during mammary gland development. In the MMTV-CR-1 transgenic mouse model, overexpression of Cripto-1 was associated with significantly increased lateral side branching of the developing mammary ducts [82]. In another transgenic model where Cripto-1 expression was driven by the Whey acidic protein promoter, the mammary glands of the transgenic mice overexpressing Cripto-1 were characterized by delayed lobuloalveolar development [83] (Fig. 4). In those studies, both transgenic models also showed increased incidence of mammary gland tumors with the predominant formation of papillary adenocarcinomas in the mammary glands of the MMTV-Cripto-1 transgenic mice and a mixture of papillary, microglandular, solid and myoepithelial histotypes containing multiple areas of squamous metaplasia in the mammary gland tumors of the WAP-Cripto-1 transgenic mice.

Overexpression of Cripto-1 cDNA in normal mouse fibroblasts induce these cells to grow in soft agar and increase growth rates in several human breast cancer cell lines [84,85]. Insertion of Elvax pellets containing the EGF-like motif of Cripto-1 protein into the mammary gland of ovariectomized virgin mice also caused increases in DNA synthesis in mammary epithelial cells in proximity to the pellets [86]. Human MCF-7 breast cancer cells that overexpress Cripto-1 proliferate at higher rates in serum-free medium, form increased numbers of colonies in soft agar, are more resistant to apoptosis when grown under anchorage independent conditions, and show increased propensity to invade and migrate *in vitro* [87]. Overexpression of Cripto-1 in the mouse mammary epithelial cell line EpH4 also caused increased cell proliferation and anchorage-independent growth in soft agar and increased chemotaxis and migration when these cells were cultured on plastic or on porous, matrix-coated filters [88]. Biochemical changes, such as reduced expression of E-cadherin or increased expression of vimentin that characterize EMT [89] were investigated in mammary gland hyperplasias and tumors from MMTV-CR-1 transgenic mice, and in the mouse mammary epithelial cell line, HC-11, overexpressing Cripto-1 (HC-11/Cr-1) [90]. E-cadherin expression was significantly decreased in tissue extracts from the mammary tumor lesions that express the human Cripto-1 transgene and in extracts from HC-11/Cr-1 cells. Those extracts also showed significant increases in the expression of N-cadherin, vimentin and integrins such as, β -4 integrin as well as increases in activated c-src, FAK and AKT, all

known to be activated during EMT and likely to play a role in increasing tumor cell invasion [89]. Also, in the Cripto-1 transgenic mammary gland tumors and HC-11/Cr-1 cells, the zinc-finger repressor transcription factor, Snail, known to down-regulate or interfere with the normal expression of E-cadherin, was detected at significantly higher levels as compared to normal control mammary tissue, thus suggesting a novel link between Cripto-1 expression and Snail activity [56].

In contrast to Cripto-1, which has been studied extensively as a mediator of breast development and tumorigenesis, evidence for a direct role for Nodal in these processes is lacking. It cannot be excluded that Nodal can play a role during mammary gland development. In fact, Nodal mRNA expression in the mouse mammary gland parallels that of Cripto-1 during different stages of development [79]. Studies analyzing mammary glands from Nodal hypomorphic mice are currently in progress and preliminary findings seem to suggest the importance of Nodal for normal mammary gland development (D.S. personal communication). These observations suggest that Nodal may play a synergistic role with Cripto-1 during the development and functional differentiation of the mammary gland.

While Nodal has not yet been overexpressed in normal or neoplastic breast cell lines, the non-tumorigenic, poorly invasive, and well differentiated human melanoma cells (C81-61) has been transfected with a vector encoding mature murine Nodal. In this cell line, overexpression of Nodal resulted in the acquisition of a tumorigenic phenotype (unpublished work). Indeed, in contrast to control C81-61 cells, which are unable to form tumors, 100% of the animals injected with Nodal-expressing C81-61 cells formed palpable tumors within 5 weeks. It will be interesting to examine if ectopic Nodal expression in poorly aggressive breast cancer cells will similarly promote tumor formation. In addition, future studies will involve the creation of transgenic animals that overexpress the Nodal transgene driven by a mammary-specific promoter, so that the role of Nodal in breast development and tumor progression may be deciphered.

CRIPTO-1 EXPRESSION IN BREAST CANCER AND POTENTIAL DIAGNOSTIC AND THERAPEUTIC APPLICATION

Cripto-1 mRNA and protein have been detected in several human breast cancer cell lines in ductal carcinoma *in situ* (DCIS) and in infiltrating breast carcinomas analyzed. In comparison, a very low percentage of normal breast specimen and adjacent non-involved breast tissue show Cripto-1 expression [47, 91–93] (Fig. 5A). In addition, significantly higher levels of Cripto-1 can be detected by immunoassay in the plasma of breast cancer patients as compared to Cripto-1 levels detected in healthy controls suggesting that Cripto-1 might be useful in the early diagnosis of this disease [94] (Fig. 5A). More recently, Cripto-1 was detected in almost half of the breast cancer tissue samples analyzed and found to correlate with advanced stage disease [95]. In addition, Cripto-1 is frequently coexpressed with other EGF-related peptides, such as TGF α , amphiregulin (AR), and heregulin in human primary breast carcinomas suggesting that Cripto-1 might cooperate with these different growth factors in supporting the autonomous proliferation of breast cancer cells. A positive correlation between nuclear *HER-4* expression and Cripto-1 expression in primary human breast carcinomas has also been described [96] further supporting the finding that Cripto-1 can indirectly enhance the tyrosine phosphorylation of *HER-4* [97].

We have recently begun to examine if Nodal expression is similarly associated with breast carcinoma progression. Like Cripto-1, Nodal is expressed in breast carcinoma cell lines but is absent in normal breast derived cells, including human mammary epithelial and myoepithelial cells (Fig. 6A & B) [98]. Using a tissue microarray we have determined that Nodal correlates with clinical breast carcinoma progression [98]. Similar to Cripto-1, Nodal

expression was absent in normal breast tissue. However, in contrast to Cripto-1, Nodal was not detected in DCIS. Rather, Nodal expression was observed later in more advanced invasive (IDC) and metastatic lesions (Figure 6C-F Nodal) [98]. This finding is comparable to that observed in melanoma specimens [23]. For example, Nodal was not detected in normal skin or in normal melanocytes, and was also absent in non-invasive radial growth phase (RGP) melanomas. Nodal was, however, detected in the invasive vertical growth phase (VGP) lesions and was present in over 60% of melanoma metastases [23]. Collectively, these results suggest that the acquisition of Nodal expression is associated with metastatic competence, which is obtained during the transition of a non-invasive cancer, such as DCIS or RGP melanoma, to an invasive lesion, such as IDC or VGP melanoma. Data generated to date with aggressive melanoma cells indicate less than 2% Cripto-1 positivity within the heterogeneous cell lines [98]. Future studies will examine whether this subpopulation of Cripto-1 positive cells can potentiate Nodal expression, and if these two molecules, independently or in co-operation, can facilitate metastatic disease.

Since high expression of Cripto-1 can be detected in human cancers, as compared to normal tissues, this signaling pathway might represent a target for cancer therapy. This is supported by findings describing the use of antisense oligonucleotides that reduce Cripto-1 expression and cause significant reduction of cell proliferation *in vitro* [99]. In addition neutralizing antibodies against Cripto-1 [47] were able to significantly inhibit tumor cell growth in two xenograft models with testicular (NCCIT) and colon (GEO) cancer cells that express very high levels of Cripto-1 (Fig. 5B). Moreover, rat monoclonal antibodies directed against the EGF-like domain of the Cripto-1 peptide also produced a significant inhibition of *in vitro* and *in vivo* growth of colon cancer and leukemia cells [99]. These results suggest that similar approaches for inhibiting cancer growth by interfering with expression or function of Cripto-1 may also have beneficial effects in Cripto-1 expressing breast cancer.

Recent studies have demonstrated that Nodal may also be an excellent therapeutic target for breast cancer treatment. Similar to Cripto-1, Nodal expression is largely restricted to embryonic stem cells and reproductive tissues including the testes, ovary and placenta [22,98]. Studies in our laboratory have shown that Nodal is also essential for tumorigenesis [22,23, 98]. For example, using an orthotopic mouse model, we determined that knocking down Nodal expression in human breast carcinoma cells (MDA-MB-231) with Nodal targeting Morpholinos (MO^{Nodal}) significantly mitigates the ability of these cells to form tumors (Fig. 7A). In order to establish a mechanism for the reduction in tumorigenicity, we have since examined the effects of this treatment on *in vivo* tumor cell proliferation and apoptosis [98]. Using immunohistochemical staining for Ki67 as a measure of proliferation, and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) as a measure of apoptosis, we determined that inhibition of Nodal expression with MO^{Nodal} decreases proliferation and increases apoptosis in orthotopic breast tumors (Fig. 7B). These *in vivo* data support a role for Nodal in the maintenance of breast cancer tumorigenicity and implicate the potential involvement of apoptotic pathways.

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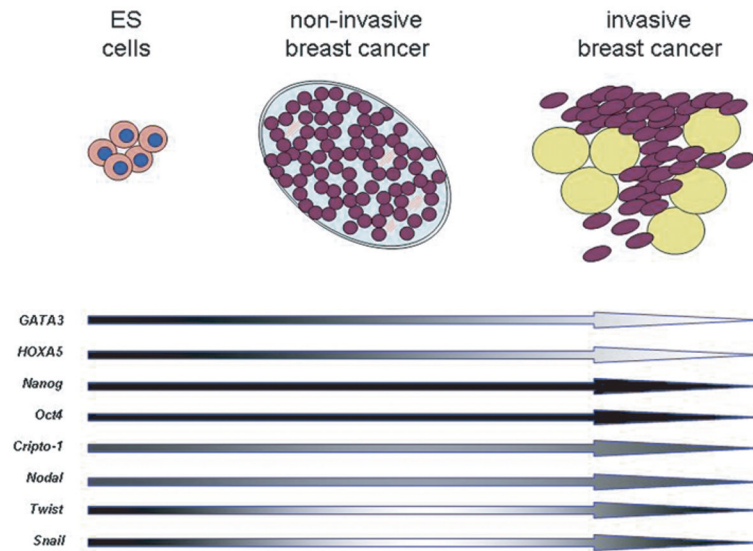


Fig. 1. Hypothetical Evolution of Stem Cell Biomarkers. Changes in expression of common embryonic stem (ES) cell related molecules in mammary epithelial cells as they transform from normal cells to non-invasive and invasive cancer cells. (Dark shade = high expression; light shade = low expression).

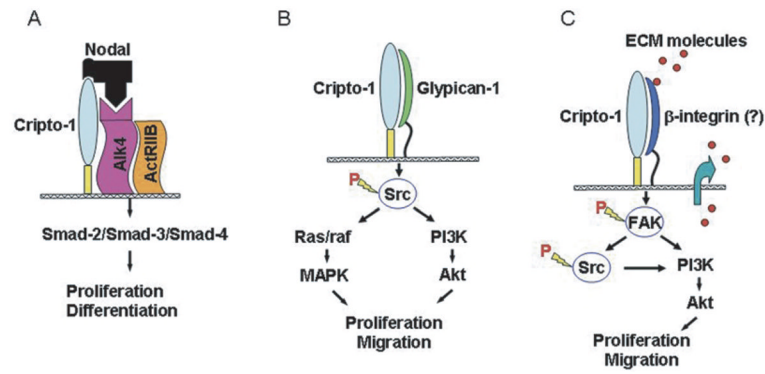


Fig. 2. Schematic Overview of Nodal and Cripto-1 Signaling Pathways. **A)** Canonical Nodal signaling using Cripto-1 as a co-receptor. Nodal can also activate this pathway, albeit less efficiently, independently of Cripto-1. **B)** Cripto-1 Nodal-independent signaling via binding with Glypican-1 and activating src/Ras/raf/PI3K downstream signaling. **C)** Possible signaling of Cripto-1 through binding to beta-integrins and signaling via FAK-dependent signaling pathway.

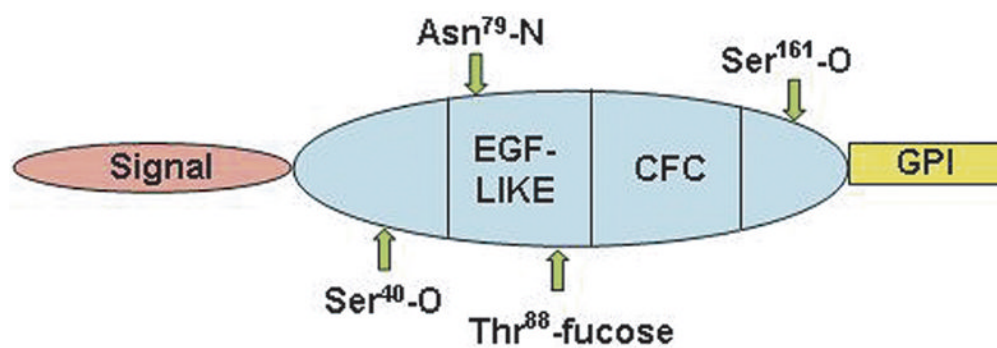


Fig. 3. Schematic Representation of Cripto-1 Protein Sequence. Note positions of the EGF-like and CFC domains which bind Nodal and ALK4, respectively.

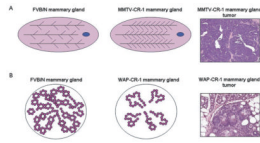


Fig. 4. Effects of Cripto-1 Transgene Expression in the Mouse Mammary Gland. **A)** Overexpression of human Cripto-1 in MMTV-CR-1 transgenic mice is associated with increased lateral side branching of mammary ducts and increased incidence of mammary papillary adenocarcinoma in multiparous mice. **B)** Overexpression of Cripto-1 in WAP-Cripto-1 transgenic mice is associated with delayed lobuloalveolar development and increased incidence of mammary gland tumors with variable histotypes including multiple areas of squamous metaplasia.

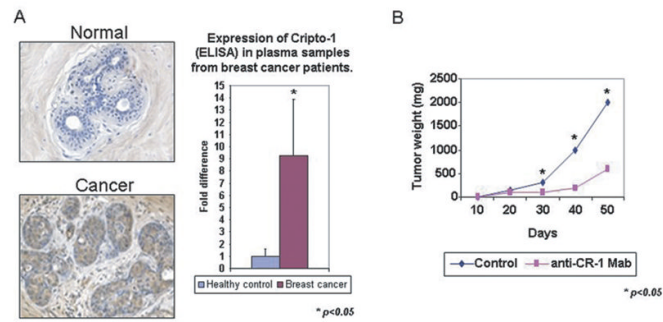


Fig. 5. Potential for Cripto-1 as a Biomarker for Cancer and Therapeutic Target. **A)** Cripto-1 is expressed in breast cancer tissue and in plasma samples from breast cancer patients. **B)** Effect of anti-Cripto-1 monoclonal blocking antibody on growth of NCCIT human teratocarcinoma cells *in vivo*. Anti-Cripto-1 blocking antibody negatively affects growth of NCCIT in a xenograft model.

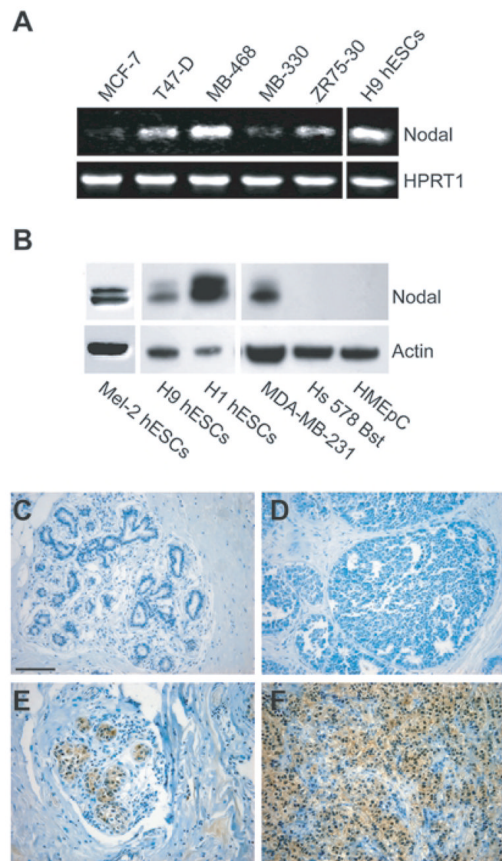


Fig. 6. Nodal Expression Correlates with Breast Carcinoma Progression. **A)** Semiquantitative RT-PCR analysis of *Nodal* mRNA expression in: MCF7, T47D, MDA-MB-468, MDA-MB-330, and ZR75-30 (human breast carcinoma cells). H9 hESC mRNA is used as a positive control for gene expression and HPRT1 is used as a loading control. **B)** Western blot analysis of Nodal in: MEL-2, H1 and H9 human embryonic stem cells (hESCs); MDA-MB-231, human metastatic breast carcinoma cells; Hs 578 Bst normal human myoepithelial cells; and HMEpC normal human mammary epithelial cells. Actin is used as a loading control. **C)** Immunohistochemical analysis of Nodal staining (dark color) in normal breast tissue, ductal carcinoma *in situ* (DCIS), nonspecific invasive ductal carcinoma (IDC) and metastatic IDC (to lymph nodes). The expression and prevalence of Nodal staining in breast tissue was designated as none, weak (<25%), moderate (25–75%) or strong (>75%). Spearman's rank correlation showed a significant positive correlation between breast cancer progression and Nodal expression ($p < 0.05$). Bar equals 100 μm for all representative images shown.

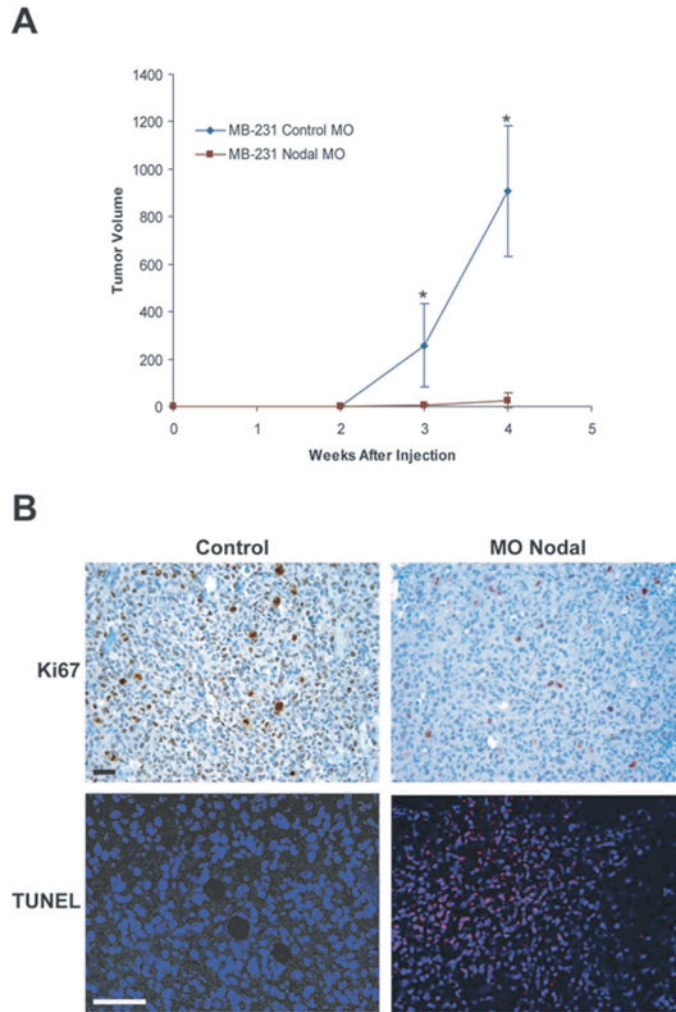


Fig. 7. Nodal Inhibition Diminishes Breast Carcinoma Tumorigenicity in an Orthotopic Model. **(A)** *In vivo* tumor formation in a mouse injected orthotopically with MDA-MB-231 human breast carcinoma cells treated with either a control morpholino (MO^{Control}) or a morpholino targeting Nodal (MO^{Nodal}) ($n = 10$). Values represent the mean tumor volume (mm³) \pm standard deviation, and tumor volumes were significantly different at the time points indicated by an asterisk (*) ($P < 0.05$). **(B)** Immunohistochemical analysis of Ki67 expression (dark) and TUNEL (light spots) staining in orthotopic breast carcinoma (MDA-MB-231) tumors. Prior to injection into a mouse, cells were treated with MO^{Nodal} or left untreated (control). Proliferation is indicated by Ki67 staining and apoptotic nuclei were detected with confocal microscopy as light staining localized to the nuclei of apoptotic MDA-MB-231 cells. Bar equals 25 μ m.