Review

Cancer Research

Nodal Expression and Detection in Cancer: Experience and Challenges

Luigi Strizzi[1], Katharine M. Hardy[1], Dawn A. Kirschmann[1], Lars Ahrlund-Richter[2], and Mary J.C. Hendrix[1]

Abstract

Nodal is a TGF-β–related embryonic morphogen that is expressed in multiple human cancers. Detection of Nodal expression in these tissues can be challenging if issues related to Nodal transcription and protein processing are not considered. Here, we discuss certain characteristics related to Nodal expression and function and how these can facilitate acquisition and interpretation of expression data, contributing to our understanding of the potential role of Nodal in human cancer. We also discuss how Nodal could be exploited clinically as a novel biomarker for cancer progression and therapeutic target. *Cancer Res; 72(8); 1915–20. ©2012 AACR.*

Introduction

Cancer cells can exploit normally dormant embryonic pathways to promote tumorigenicity and metastasis. Understanding the impact of these embryonic signals and the regulatory programs that reactivate them holds significant potential for new cancer therapies. Studying embryonic signaling pathways in cancer, particularly aggressive cancer, has led to the discovery of the reexpression of the embryonic morphogen Nodal (1). Nodal is a member of the TGF-β superfamily, essential in maintaining the pluripotency of human embryonic stem cells (hESC). Recent findings have revealed that Nodal is a critical regulator of melanoma growth, plasticity, and tumorigenicity, and that it holds promise as a new biomarker for metastatic potential (1–3). Similar observations have been reported in gliomas and carcinomas of the breast, endometrium, and prostate (4–7).

Nodal is an important regulator of early vertebrate development, including mesoderm formation, body plan establishment, and cell fate determination (8). In humans, Nodal expression is largely restricted to embryonic tissues, including the trophoblast and the developing mammary gland, but is generally lost in normal adult tissues (4). Therefore, studies addressing the role of Nodal in cancer progression have focused on the mechanisms underlying its reexpression in tumor cells and the translational relevance of targeting Nodal as a novel therapy (9).

With any new discovery, there are associated challenges. As investigators introduce novel findings to the literature, it is with the expectation that other scientists will confirm and extend their findings. In the case of Nodal, this process has been particularly challenging and confounding due to inconsistencies and sometimes incorrect information available in public databases, in addition to lackluster reagents for human cell studies. This review is dedicated to full transparency and disclosure of some of our challenges and experiences related to the study of Nodal.

Processing and Signaling of Nodal

Much of our understanding of how Nodal protein is processed and propagates signaling comes from studies related to developmental biology, because Nodal is a critical factor in normal embryonic development and regulates numerous developmental processes, including gastrulation and left–right asymmetry (8, 10, 11). Canonical Nodal signaling is propagated via the binding of Nodal ligand to the Cripto-1 coreceptor and a complex of type I and type II activin receptors (ALK4/7 and ActRIIB, respectively), triggering phosphorylation events that activate Smad2/3 and facilitate binding to Smad4 (Fig. 1A; ref. 11). This Smad complex associates with other transcription factors in the nucleus and propagates the transcription of target genes including Nodal itself and the Nodal antagonist, Lefty. Under normal circumstances, the positive feedback on Lefty expression, as well as Nodal, serves to limit signaling activity and provides a more refined level of pathway regulation. However, in cancer cells studied, the Lefty gene is highly methylated and does not respond to Nodal signaling, allowing Nodal transcription to proceed unchecked (4, 12). Exposing tumor cells to Lefty produced by hESCs dramatically inhibits Nodal expression and reduces clonogenic potential (4).

Nodal signaling can occur in both an autocrine and paracrine fashion and may be influenced by the processing, stability, and trafficking of Nodal protein (10, 11). Nodal is translated in a precursor form consisting of a signal peptide, prodomain, and mature domain. Transfection studies with exogenous mouse Nodal suggest that the proform (pro- and mature domains) is cleaved to a much less stable, but highly active mature form extracellularly by the proprotein

Authors' Affiliations: [1]Children's Memorial Research Center, Cancer Biology and Epigenomics Program, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois; [2]Karolinska Institute, Department of Women's and Children's Health, Stockholm, Sweden

Corresponding Author: Mary J.C. Hendrix, Children's Memorial Research Center, 2300 Children's Plaza, Box 222, Chicago, IL 60614. Phone: 773–755-6528; Fax: 773–755-6534; E-mail: m-hendrix@northwestern.edu

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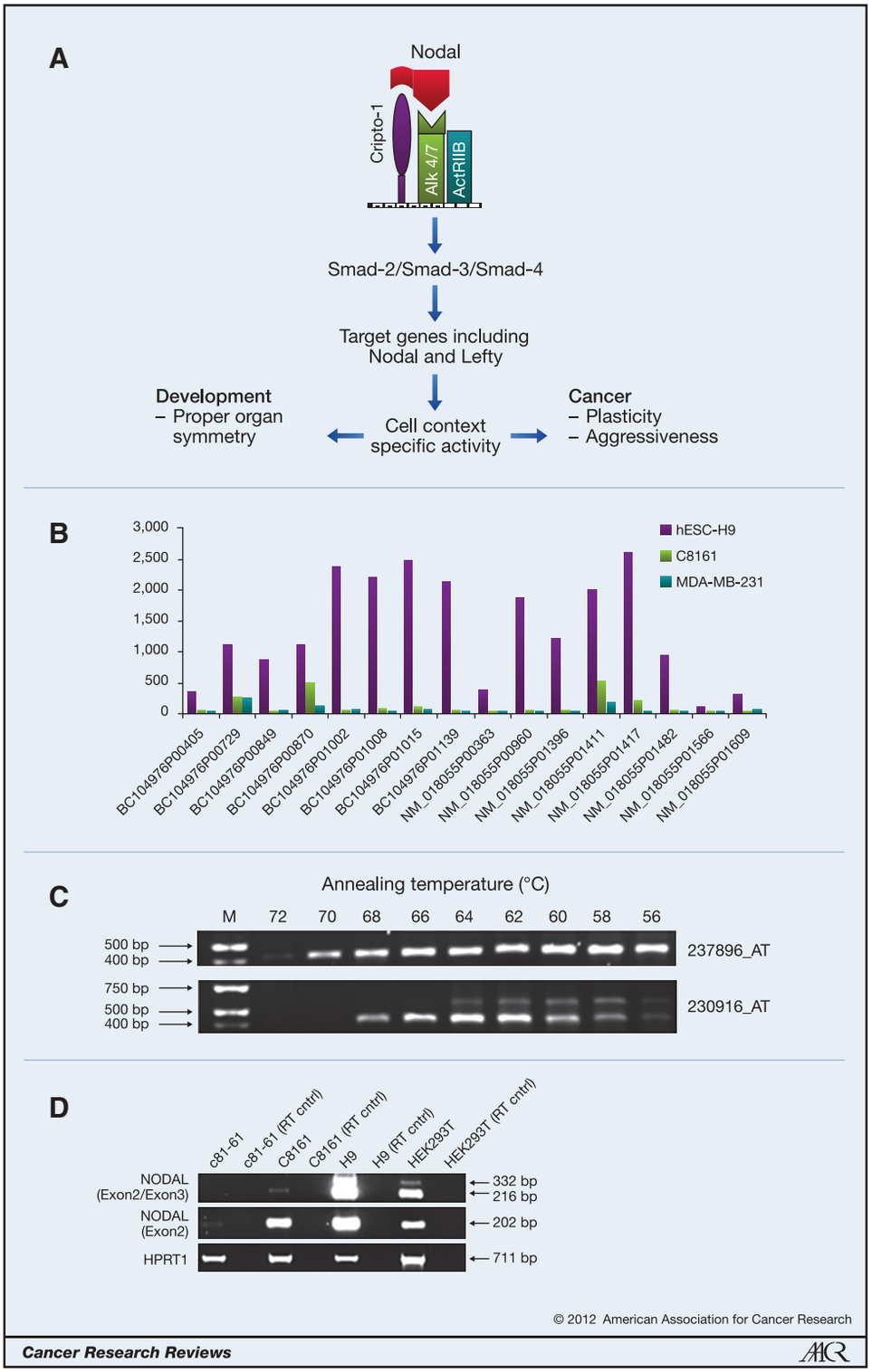

Figure 1. A, schematic representation of primary Nodal signaling events. B, microarray results (NimbleGen HG18 chip) of mRNA from hESC-H9, melanoma (C8161), and breast cancer (MDA-MB-231) cell lines, showing wide variability in detection levels among 16 different probes all designated as recognizing *Nodal*. C, results from PCR experiments show that primers corresponding to the Affymetrix U133B chip 237896_AT microarray probe produce a single PCR product that corresponds to the *Nodal* gene, whereas sequencing of the 2 PCR products recognized by the 230916_AT probe does not correspond to the *Nodal* gene. D, semiquantitative PCR analyses in human embryonic stem cells (H9), and human embryonic kidney cells (HEK293T), poorly aggressive (c81–61), and highly aggressive (C8161) human melanoma cells with primers spanning the exon 2/exon 3 boundary identify 2 bands that may indicate splice variants of *Nodal*. RT cntrl, no reverse transcriptase present.

convertases Furin and Pace4 (10). Certainly, in mice, Furin and Pace4 are required for Nodal signaling (13). Transfection studies also suggest that Cripto-1 could further regulate maturation by anchoring the proform of mouse Nodal and 1 of the proprotein convertases in a complex at the plasma membrane and may facilitate Nodal internalization as well as signaling (10); however, whether this has relevance in cancer is not yet clear. Nodal can signal independently of Cripto-1 (11), and, in melanoma cell lines, endogenous Cripto-1 is present on the surface of only a small percentage (less than 5%) of cells (4, 14), suggesting the possibility that a canonical mechanism of signaling may not necessarily occur in cancer cells. In tumor cells, it is also not clear if Nodal signals in a paracrine or autocrine fashion or both; although, by immunofluorescence microscopy, Nodal protein is observed in only a proportion of melanoma cells in culture (29%–39% depending on the cell line), suggesting that the signaling range of Nodal is limited, but may also identify an important cellular subpopulation (15). Indeed, more research is needed to determine the precise mechanisms that regulate Nodal signals at the single cell level in cancer cells.

Multiple groups have now detected human Nodal protein in hESCs and various cancer cell lines with commercially available antibodies from different companies, in assays including Western blotting, immunofluorescence, immunohistochemistry, and immunoprecipitation (1, 3, 7, 15). In particular, Western blot analyses in human cancer cells indicate that the predominant species of endogenous Nodal protein are the precursor and proforms, though reported molecular weights vary (42,000–48,000 and 35,000–37,000, respectively; refs. 1, 3, 4, 7, 15). The mature form (~13,000–15,000) is rarely observed, suggesting it is likely highly unstable and/or cleaved predominantly outside the cell. However, what is clear in our experience is that particular care must be taken in preparing and storing protein lysates, as Nodal protein (especially the mature form) is highly susceptible to degradation, leading to variable levels of detection over time, even between aliquots of the same sample.

Considering that Nodal is not typically expressed in adult tissues, one important question has been how Nodal is upregulated in cancer. Recent research suggests that Nodal reexpression in aggressive melanoma is regulated by a Notch signaling pathway (15). Certainly, a molecular link between these 2 prominent stem cell–associated pathways exists, as shown by the *in vitro* activity of an RBPJ-dependent Nodal enhancer element. Specifically, the expression of Notch4 and Nodal was observed to correlate in aggressive melanoma cell lines, but not poorly aggressive cell lines, as well as in advanced-stage melanomas. Targeting Notch4 expression or activity downregulated Nodal levels in aggressive cell lines, and exogenous expression of the Notch4 intracellular domain in poorly aggressive cells upregulated Nodal levels, indicating Notch4 specifically regulates the Nodal gene in melanoma cell lines. *In vitro* biologic assays of vascular-like network and colony formation were perturbed by Notch4 inhibition and could be partially rescued by recombinant Nodal, suggesting melanoma aggressive cell behavior is controlled at least in part by Notch4 regulation of Nodal. Whether Nodal expression is regulated by a Notch signaling pathway in other cancers is not yet known.

Expression and Detection of the Nodal Gene: Lessons Learned

Exhaustive investigations related to human Nodal expression and function in cancer have proven challenging, particularly with regard to gene expression studies. In fact, few studies have reported substantive analyses of *Nodal* gene expression in human cancer cells (3, 4, 15). Efforts by our group to detect a *Nodal*-specific amplicon in human cancer cells, and in some cases also hESCs, using primers previously published in studies with human trophoblast, breast cancer, and hESC lines (16–18), were met with limited success. The reason for this discrepancy is not entirely clear, though certainly, *Nodal* expression in cancer cell lines is less abundant than in embryonic cells (4), which may account for some detection differences. Importantly, verification of a correlation between mRNA and protein in cancer cells has been achieved, but only when selecting semiquantitative primers located in either exon 2 or the 3′-untranslated region of the human *Nodal* gene sequence or with a commercially available real-time PCR primer probe set (Applied Biosystems Hs00250630_s1) also located in exon 2 (15). As such, extensive controls to exclude detection of contaminating genomic DNA (DNAse treatment and omission of reverse transcriptase in control samples) are necessary when evaluating human *Nodal* mRNA levels with these tools. Notably, the only published report of human *Nodal* mRNA detection by *in situ* hybridization was achieved in human tumor xenograft tissue sections with an antisense RNA probe complementary to a portion of exon 2, and, in this instance, extensive signal amplification with a biotin-streptavidin tyramide signal amplification kit was necessary (3). Together, these findings indicate how important primer and sequence selection is in elucidating human *Nodal* gene expression, particularly in tumor cells.

Probes for the human *Nodal* gene used by manufacturers of microarray chips show considerable variability in detecting *Nodal* expression levels. Levels of gene expression in hESC-H9 and cancer cell lines C8161 and MDA-MB-231 were evaluated by our laboratory using the HG18 Human Gene Expression 385K Microarray Chip (NimbleGen, Roche Applied Sciences). Of the 16 probes designated as Nodal, individual detection levels were widely inconsistent, ranging from 305.4 to 2,630.63 relative fluorescent units in hESC-H9s (Fig. 1B). Furthermore, it is not clear that all probes designated as Nodal actually specifically detect *Nodal* expression. The Affymetrix chip U133B employs 2 probes for Nodal (237896_AT and 230916_AT), both of which contain sequences in exon 3 of the Nodal gene. PCR amplification of these 2 regions, for 237896_AT, gave a PCR product that was sequenced and verified as *Nodal*, and for 230916_AT, gave 2 PCR products that were both repeatedly sequenced and never corresponded to the *Nodal* gene sequence (Fig. 1C), suggesting it is not specific for Nodal. Discordances such as these exemplify the possibility that *Nodal* could be incorrectly detected or disregarded as unexpressed in standard mRNA expression analyses

such as microarray. This analysis highlights the importance of verification by multiple methods, especially in detecting *Nodal* gene expression.

Confounding the situation further is the possibility that multiple Nodal splice variants may exist. Semiquantitative PCR analyses in hESCs with primers spanning the exon 2/exon 3 boundary identified 2 bands (Fig. 1D). DNA sequence analysis indicated that the novel, larger amplicon included a 116-bp region of intron 2 located at the exon 2/3 splice junction, suggesting the possibility that it represents a novel splice variant. Consistent with this, submissions in National Center for Biotechnology Information AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&term=Nodal&submit=Go) and Ensembl (http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000156574;r=10:72192071-72207707) suggest that the human *Nodal* gene may generate up to 4 mRNA species. Despite this finding, the only validated and complete *Nodal* sequence (NM_018055.4) encodes a full-length protein, detectable by Western blotting and other assays. Extensive studies would be required to determine whether these putative *Nodal* splice variants are translated into protein or are possible regulatory RNAs, and, more importantly, whether there is tissue specificity with regard to their gene expression. Remarkably, all 3 putative *Nodal* splice variants contain a complete or large portion of exon 2, which, if expressed, may offer a plausible explanation for why primers/probes in exon 2 accurately detect *Nodal* gene expression, whereas many others do not. Nonetheless, these observations show that detection of *Nodal* gene expression is complex and exemplify why extreme care should be taken when evaluating Nodal expression in human cancer and other cells.

Evidence for Clinical Potential of Targeting Nodal in Cancer

Variability in response rates among patients with the same cancer type, or even in the same patient during the evolution of a specific malignancy, is often due to the high level of heterogeneity within the cancer cell population. In particular, whereas a subset of cancer cells may be sensitive to a certain anticancer drug, others may continue to proliferate unabated, because these cells have either developed resistance to that chemotherapeutic or simply do not express the target for which the drug was developed. In melanoma, for instance, several signaling molecules or pathways have been identified as potential therapeutic targets (19). One widely studied example is the mitogen-associated protein kinase signaling pathway associated with activating mutations in NRAS or BRAF, which have recently been the focus of targeted therapy in melanoma (20). However, not all melanomas present these specific mutations or activated signaling pathways, and some develop resistance to targeted therapy. Consequently, a significant proportion of melanoma patients do not benefit from these novel targeted approaches (21). The same is true in breast cancer in which the hormone receptors (HR) estrogen and progesterone, useful biomarkers for predicting survival and therapeutic outcome, are either not expressed in all patients or are not expressed homogeneously within the same tumor of an individual patient. In fact, patients who were once HR positive may become HR negative or, as occasionally reported, HR-negative patients can revert to HR positive, affecting the efficacy of targeted treatment (22). The ideal molecular target, therefore, would be detected in specific cancer cells responsible for disease progression and, most importantly, expressed in all affected patients. An alternative to this "magic bullet" approach would be to increase our armamentarium of inhibitors of molecular targets by identifying additional, functionally relevant targets, leading to a multitargeted approach to eliminate more subsets of the total heterogeneous cancer cell population.

Studies from cancer stem cell (CSC) research have suggested that targeting cancer cells with stem cell–like characteristics, which seem to be responsible for the morphologic and functional heterogeneity seen in cancer, could affect survival by reducing tumor growth, metastatic spread, drug resistance, and disease recurrence (23). Advances in the field of CSC research have enabled us to characterize the reemergence of specific embryonic signaling pathways, such as Nodal, thus contributing to our understanding of the molecular mechanisms that regulate cancer cell plasticity and aggressiveness (24). Because Nodal expression is not generally detected in normal tissues, but can reemerge in a number of human cancers including melanoma, breast, and prostate cancer (1, 4, 7), Nodal could represent a candidate target in aggressive cancer cells.

Our work has shown that downregulation of Nodal can be achieved, either by directly targeting Nodal with Nodal Morpholino or by exposing cells to hESC-derived Lefty, or by targeting Notch4 upstream of Nodal expression, thus reducing tumorigenicity, plasticity, and aggressiveness of human melanoma and breast cancer cells (1, 4, 15). In addition, Nodal-specific short hairpin RNA has been shown to reduce Nodal expression and decrease invasiveness and tumor growth of human glioma cells, both *in vitro* and *in vivo* (6). Given the complexity of Nodal detection, as previously described in this review, it must be noted that Nodal knockdown experiments must be validated by protein expression analysis, especially with RNA interference methods in which PCR detection of partially degraded, nontranslating transcripts could lead to potential misinterpretation of results (25). Certainly, tumors in Nude mice formed from injected Nodal-expressing aggressive human melanoma cells exhibited a decrease in tumor cell proliferation and increase in apoptosis when mice were treated intratumorally with hESC-derived Lefty (4). Similarly, aggressive melanoma cells injected retro-orbitally in Nude mice and allowed to colonize to the lung formed fewer tumor cell colonies when treated with intraperitoneal injections of a function-blocking anti-Nodal antibody, compared with mice injected with an isotype control immunoglobulin (26). Of note, melanoma cells observed in the lungs of antibody-treated mice showed more frequent signs of cellular distress and apoptosis compared with control mice. Collectively, these studies suggest the potential for Nodal as a target for human cancer therapy.

Implications and Future Directions

The plastic phenotype associated with aggressive tumors presents a significant challenge in the detection and targeting of cancer cells exhibiting stem cell–like characteristics. As we elucidate the embryonic signaling pathways reactivated in many cancers and their contributions to tumor cell plasticity, new strategies will emerge about the suppression of this elusive phenotype. One of these pathways, the Nodal signaling pathway, is a master regulator of tumor cell plasticity and tumorigenicity. Because Nodal is not expressed by most normal adult tissues and is overexpressed by aggressive tumor cells, it may represent a valuable new therapeutic target. The methylation (and silencing) of Nodal's inhibitor, Lefty, also provides an additional consideration for therapeutic application. Not only does exogenously added Lefty suppress Nodal expression in tumor cells, but Lefty can also be reexpressed in tumor cells treated with 5-azacytidine (D.A. Kirschmann; personal communication). Equally noteworthy is the molecular cross-talk between Nodal and Notch, via Notch4 regulation of Nodal expression. The implications of this new finding suggest that tumor cells coexpressing Notch4 and Nodal may better define the CSC phenotype. Furthermore, suppression of this phenotype may require a combinatorial multitarget approach. Using modest patient sample numbers in immunohistochemistry studies, the diagnostic potential of Nodal in human melanoma, breast, and prostate carcinoma seems promising (2, 4, 7). However, ongoing studies with ambiguous nevi will ultimately assess the prognostic and predictive power (or value) of Nodal in identifying patients at risk for melanoma disease onset and progression. Finally, this review reveals the challenges we and others have faced in accurately measuring Nodal gene and protein expression on the basis of the information available in the literature, public databases, and commercial reagents. Let us not forget that the role of Nodal in cancer is a relatively new observation and, as such, deserves continuous validation, thoughtful discussion, and patience to understand its full impact on the field.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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