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Targeting the Stem Cell Properties of Adult Breast Cancer Cells: Using Combinatorial Strategies to Overcome Drug Resistance

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Abstract

Purpose of review—Cancer is a major public health problem worldwide. In aggressive cancers, which are heterogeneous in nature, there exists a paucity of targetable molecules that can be used to predict outcome and response to therapy in patients, especially those in the high risk category with a propensity to relapse following chemotherapy. This review addresses the challenges pertinent to treating aggressive cancer cells with inherent stem cell properties, with a special focus on triple-negative breast cancer (TNBC).

Recent findings—Plasticity underlies the cancer stem cell (CSC) phenotype in aggressive cancers like TNBC. Progenitors and CSCs implement similar signaling pathways to sustain growth, and the convergence of embryonic and tumorigenic signaling pathways has led to the discovery of novel oncofetal targets, rigorously regulated during normal development, but aberrantly reactivated in aggressive forms of cancer.

Summary—Translational studies have shown that Nodal, an embryonic morphogen, is reactivated in aggressive cancers, but not in normal tissues, and underlies tumor growth, invasion, metastasis and drug resistance. Front-line therapies do not inhibit Nodal, but when a combinatorial approach is used with an agent such as doxorubicin followed by anti-Nodal antibody therapy, significant decreases in cell growth and viability occur. These findings are of special interest in the development of new therapeutic interventions that target the stem cell properties of cancer cells to overcome drug resistance and metastasis.

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Compliance with Ethical Standards

Conflict of Interest Naira V. Margaryan declares that she has no conflicts of interest. Elisabeth A. Seftor, Richard E.B. Seftor, and Mary J.C. Hendrix are listed as co-inventors on Nodal-related patents and/or disclosures.

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Keywords

Nodal; Cancer stem cells; Breast cancer; Doxorubicin; Combinatorial therapy; Predictive biomarker

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Introduction

Aggressive cancer cells are characterized by their ability to proliferate indefinitely and to propagate a heterogeneous tumor inclusive of cells with metastatic propensity and drug resistance properties. Seminal studies in cancer research have allowed scientists to identify and isolate tumor-initiating stem cells within these diverse subpopulations, contributing to the cancer stem cell (CSC) theory [1]. Indeed, one of the greatest challenges we face in the field of oncology is the effective targeting of heterogeneous tumors containing varied subpopulations of cancer cells expressing a variety of markers, especially those associated with the stem cell phenotype. This is particularly true in breast cancer where myriad classification schemes have been developed based on histological criteria [2], differentiation status [3], and molecular markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [4]. The latter have proven to be valuable for stratifying certain types of breast cancers with respect to treatment options and outcome predictions. More recent classification analyses based on gene expression microarrays have resulted in molecular-based subgroups, such as luminal and non-luminal breast tumors [5], in addition to CSC subpopulations identified by the CD44^{high}/CD24^{low} molecular profile [6]. Although these findings are considered significant and have advanced our thinking about classifying different risk groups relevant to more effective targeting, we recognize that heterogeneity within various subgroups exists which continues to confound our ability to completely eradicate breast cancer progression, relapse and metastasis in many cases, such as aggressive triple-negative breast cancer (TNBC) which exhibits little to no expression of classical markers.

Tumor cell heterogeneity, as depicted in Figure 1, is illustrated by the basic steps that occur from the time of an initial diagnosis through metastasis. In this scheme, using TNBC as an example, the primary tumor is comprised of heterogeneous subpopulations; the biopsy sampled from the primary tumor may or may not contain all the subpopulations represented. However, histological assessment and biomarker identification lead to the initial diagnosis and treatment decision. The front-line therapy reduces the bulk of the primary tumor, but residual disease may exist which results in relapse and metastatic disease. The expansion of specific subpopulations, including CSCs, not targeted by the front-line therapy comprise the metastatic lesion, from which a second diagnosis occurs and subsequent second-line therapy administered. Further expansion of specific subpopulations, such as CSCs, resistant to second-line therapy populate additional metastatic lesions and further dissemination of breast cancer.

The aim of this review is to present information that highlights the importance of tumor cell plasticity underlying the CSC phenotype in breast cancer, with a particular focus on the re-emergence of the Nodal embryonic signaling pathway and its association with

tumorigenesis, metastasis and drug resistance to front-line therapy. Nodal is a promising prognostic and predictive biomarker in breast cancer, as well as a target for therapeutic intervention [7•, 8•, 9•]. These findings may help inform the development of combinatorial approaches to target CSC subpopulations in aggressive cancers, such as TNBC.

Tumor Cell Plasticity

Our basic understanding of the phenotype and functional properties of cancer cells has been greatly enhanced by the use of molecular tools generating gene expression profiles to help characterize specific risk groups [10•, 11, 12•]. Select microarray analyses of aggressive breast cancer cells compared with nonaggressive breast cancer reveals the noteworthy co-expression of multiple cellular phenotype markers associated with endothelial cells, mesenchymal cells and stem cells concomitant with the down-regulation of breast epithelial markers (Table 1). At first glance these data seem confounding; however, further functional analyses have revealed unique insights into the plasticity of the aggressive tumor cell phenotype and selective advantages imparted to tumorigenesis, metastasis and drug resistance. For example, tumor cells with this type of molecular profile appear to dedifferentiate from their original epithelial cell origin and transition to a more mesenchymal phenotype, generally referred to as epithelial-to-mesenchymal transition (EMT). Also noteworthy, the up-regulation of endothelial cell markers by aggressive breast cancer cells contributes to vasculogenic mimicry (VM), a relatively new paradigm in cancer biology, which describes the formation of perfusable, vasculogenic-like networks lined by tumor cells -- advantageous for growth and metastasis [13•]. Most importantly, angiogenesis inhibitors do not target VM, which has prompted the pharmaceutical industry to develop a different class of vascular disrupting agents [14•]. Examples of VM have been reported across a wide spectrum of tumor types, including carcinomas, melanoma, sarcomas, gliomas, and neuroblastomas [for review, 13•, 15]. Further to this point, a recent study using sophisticated molecular tracing of heterogeneous breast cancer cells -- studied in a primary tumor that ultimately metastasized -- identified two proteins, Serpine 2 and Slpi, that enable tumor cells to form VM networks, which in turn facilitates perfusion and metastasis [16•].

As certain events in tumor formation seem to recapitulate developmental processes, the co-expression of stem cell markers by aggressive breast cancer cells is not surprising (Table 1). Since considerable work has been reported regarding the significance of aldehyde dehydrogenase (ALDH1), CD44, and Notch [6, 17•, 18–21], our laboratory dedicated investigative efforts on an understudied area focused on Nodal, an embryonic stem cell marker that heretofore had not been reported as a critical factor in cancer. Our knowledge base for Nodal was derived primarily from developmental biology studies, where Nodal is described as a powerful embryonic morphogen belonging to the transforming growth factor β (TGF β) superfamily and playing a critical role during embryogenesis to coordinate body axis formation, L-R patterning, and activation of EMT [22, 23]. Nodal also plays a quintessential role in maintaining the pluripotency of human embryonic stem cells (hESCs) [24]. Nodal signals *via* binding to Cripto-1/ALK4/7/ActRIIB receptor complex, leading to the downstream phosphorylation of Smad2/Smad3 and association with Smad4, and subsequent translocation to the nucleus [25•]. In humans, Nodal expression is largely restricted to embryonic tissues, and is generally lost in normal adult tissues, making it a

promising target specific to cancer. Also noteworthy, a natural inhibitor of Nodal is Lefty, a member of the TGF β superfamily [26•], which plays a prominent role in the regulation of Nodal expression during development. A comparative analysis of Nodal and Lefty in hESCs and aggressive tumor cells (breast cancer and melanoma) revealed that Nodal is strongly expressed in hESCs and aggressive tumor cells, but Lefty is only found in hESCs and is absent in cancer [27••, 28, 29•]. In fact, Lefty is silenced via methylation in cancer cells [30]. This significant finding indicates that the reactivation process underlying the embryonic Nodal signaling pathway in aggressive cancer occurs without commensurate activation of an inhibitor, thus allowing Nodal to signal in a highly unregulated manner. Furthermore, experimental studies have demonstrated the important role Nodal plays in cancer progression. Specific down-regulation of Nodal signaling in multiple cancer models results in decreased tumorigenicity, metastasis, invasion, clonogenicity, angiogenesis, and the plastic phenotype [7••, 8•, 31–33]. Because Nodal is critical to sustaining the pluripotent phenotype in hESCs, we have postulated that it is a master plasticity gene in cancer and refer to it as a CSC signaling molecule worthy of additional scrutiny and consideration.

Nodal: A Promising New Target

Translational studies have shown the immunohistochemistry (IHC) localization of Nodal in patient tissues to be associated with disease progression in a variety of tumors, including carcinomas of the breast and prostate, and in melanoma [34, 35•]. Other studies have reported the presence of Nodal in pancreas, bladder, ovary, colon, neuroblastoma, and glioblastoma [36]. From a clinical perspective, TNBC is considered a challenging disease to manage with few established targeted protocols to deploy [37–39]. Recent findings from our laboratory have revealed that Nodal protein is highly expressed in TNBC when compared directly with hormone receptor(s) (HR) and HER2 positive invasive breast cancer [9••]. A previous study of Nodal localization in 431 therapeutically naïve patients diagnosed with benign or malignant disease suggested a potential role for Nodal as a new prognostic and predictive biomarker for disease progression when compared with currently used reference markers [7••]. Collectively, these observations prompted the translationally relevant question whether Nodal is targeted by front-line therapies. Interestingly, in melanoma models, we discovered that conventional therapy utilizing dacarbazine or targeted therapy with BRAF inhibitors does not diminish Nodal expression [40•, 41••]. However, these studies further revealed that down-regulation of Nodal expression concomitant with chemotherapy was most effective in inducing tumor cell apoptosis and inhibiting metastasis. These observations are further supported by studies in pancreatic cancer, where inhibition of Nodal signaling enhances the effects of chemotherapy [42].

In alignment with these previous studies, our observations in breast cancer demonstrated that in three TNBC models, where Nodal expression is robust, treatment with the anthracycline doxorubicin (DOX) did not effectively target Nodal. However, sequential treatment of these models with DOX, followed by anti-Nodal antibody therapy, resulted in significant decreases in cellular growth and viability [9]. Additional analyses revealed that inhibition of Nodal following DOX resulted in an increase in early and/or late stage apoptosis when compared to DOX or anti-Nodal treatments alone. Further investigation of the mechanism(s) underlying this important finding took into account that DOX acts by reducing the integrity

of DNA within cancer cells. With this in mind, two stress/survival related pathways were further examined, which showed that anti-Nodal antibody treatment, following DOX, affects the cellular stress (p38) and repair (Chk1) pathways in TNBC. These findings suggest a unique role for Nodal in response to cellular damage, where inhibition of this CSC signaling molecule interferes with the cancer cell's ability to repair their compromised DNA.

Conclusions

In aggressive cancers, such as TNBC, where a lack of targetable molecules exists, together with a propensity for relapse following chemotherapy, additional studies are needed to identify novel biomarkers, based on the molecular underpinnings of disease progression. These markers can be used to predict outcome and response to therapy in patients, especially those in the high risk category. The challenges posed by heterogeneous cell subpopulations comprising a tumor, especially CSCs, remain a key focus in cancer research which can inform the design of more effective clinical trials.

We have discovered that conventional therapies do not target tumor cells expressing the embryonic morphogen Nodal, which has profound implications in cancer progression. However, experimental studies from our laboratory and others are demonstrating the promise of using a combinatorial approach consisting of a front-line therapy together with anti-Nodal treatment. Front-line therapies can effectively reduce the bulk of a tumor, followed by specific therapies that target chemoresistant CSC subpopulations, such as those expressing Nodal and other stem cell associated markers.

Progenitor cells and CSCs use similar signaling pathways to sustain growth. Observations related to the convergence of embryonic and tumorigenic signaling pathways have led to the intriguing paradigm of oncofetal targets, rigorously regulated during normal development, but aberrantly reactivated in aggressive forms of cancer [8, 43]. Particularly promising are those oncofetal targets, such as Nodal, that reemerge only in aggressive cancers and not expressed in normal tissues. These findings are of special interest in the development of new therapeutic interventions that target the stem cell properties of adult cancer cells, especially as part of a successful armamentarium in combinatorial strategies to overcome drug resistance.

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Fig. 1.

Schematic illustration of tumor cell heterogeneity in an aggressive cancer. Using triple negative breast cancer (TNBC) as an example, the primary tumor is comprised of heterogeneous subpopulations of cells. Generally, a diagnosis from a primary tumor biopsy is based on a sample of the cellular makeup from a small portion of the tumor mass. Analysis of the cellular composition of the biopsy reveals biomarkers which inform the selection of possible front-line therapies suited for treating the tumor. With reduction in the mass of the tumor, cells unaffected by the initial treatment remain and can lead to a relapse and metastasis. Additional biopsies can then inform second-line treatment regimes. Of note, cancer stem cells (CSCs), such as those expressing the embryonic morphogen Nodal, that are present in the primary tumor, can expand and demonstrate multi-drug resistance and lead to progression and metastasis of the tumor.

Table 1

Molecular profile of aggressive breast cancer cells: Tumor cell plasticity

Gene	Function	Expression
DnaJ (DNAJB9) Hsp40 homolog; Microvascular endothelial differentiation gene 1	Microvascular endothelial cell molecule	Up-Regulated
Endothelial differentiation-related factor 1 (EDF1)	Endothelial differentiation factor	Up-Regulated
Endothelial cell adhesion molecule (ESAM)	Endothelial adhesion molecule	Up-Regulated
VEGF-A VEGF-C	Endothelial growth factor	Up-Regulated
OB-cadherin (CDH11)	Osteoblast adhesion molecule	Up-Regulated
N-cadherin (CDH2)	Neuronal adhesion molecule	Up-Regulated
Laminin 5	Extracellular matrix	Up-Regulated
Fibronectin 1 (FN1)	Extracellular matrix	Up-Regulated
Vimentin (VIM)	Mesenchymal intermediate filament	Up-Regulated
Aldehyde dehydrogenase (ALDH1)	Cancer stem cell marker	Up-Regulated
CD44	Cancer stem cell marker	Up-Regulated
Nodal	Embryonic stem cell marker	Up-Regulated
Notch	Stem cell marker	Up-Regulated
MMPs	Matrix metalloproteinases	Up-Regulated
E-cadherin	Epithelial cadherin molecule	Down-Regulated
Estrogen Receptor (ESR1)	Breast hormone receptor	Down-Regulated
Progesterone Receptor (PGR)	Breast hormone receptor	Down-Regulated

Changes in gene expression level of highly aggressive compared to nonaggressive breast cancer cells with greater than a 3-fold increase (Up-Regulated) or decrease (Down-Regulated) as assayed on NimbleGen oligonucleotide microarrays. The data show co-expression of phenotypes associated with endothelial cells, mesenchymal cells and stem cells; down-regulation of breast epithelial markers