Mini-Review

Role of Cripto-1 in Stem Cell Maintenance and Malignant Progression

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Cripto-1 is critical for early embryonic development and, together with its ligand Nodal, has been found to be associated with the undifferentiated status of mouse and human embryonic stem cells. Like other embryonic genes, *Cripto-1* performs important roles in the formation and progression of several types of human tumors, stimulating cell proliferation, migration, epithelial to mesenchymal transition, and tumor angiogenesis. Several studies have demonstrated that cell fate regulation during embryonic development and cell transformation during oncogenesis share common signaling pathways, suggesting that uncontrolled activation of embryonic signaling pathways might drive cell transformation and tumor progression in adult tissues. Here we review our current understanding of how *Cripto-1* controls stem cell biology and how it integrates with other major embryonic signaling pathways. Because many cancers are thought to derive from a subpopulation of cancer stem-like cells, which may re-express embryonic genes, *Cripto-1* signaling may drive tumor growth through the generation or expansion of tumor initiating cells bearing stem-like characteristics. Therefore, the *Cripto-1*/Nodal signaling may represent an attractive target for treatment in cancer, leading to the elimination of undifferentiated stem-like tumor initiating cells. (*Am J Pathol* 2010, 177:532–540; DOI: 10.2353/ajpath.2010.100102)

Embryonic development involves coordinated processes of proliferation of progenitor stem cells that carry the potential of self-renewal and subsequent differentiation into distinct cell lineages.¹ After fertilization, totipotent stem cells of the blastocyst give rise to all tissues. With subsequent cell divisions, stem cells retain their self-

renewal capacity, but they become more restricted in their differentiation potential, becoming progenitor cells (adult or somatic stem cells) that give rise to differentiated somatic cells in specific tissues.¹

Therefore, two fundamental properties characterize stem cells: self-renewal, the ability to maintain their identity through a long period of time, and multipotency, the ability to generate all differentiated cell types of a specific tissue. A stem cell that asymmetrically divides can generate a new stem cell and a committed daughter cell. In the adult, a pool of stem cells resides within specific microenvironments or niches in adult tissues and functions as an internal repair system, dividing to replenish specialized cells and also maintaining the normal turnover of regenerative organs, such as blood, skin, or intestinal epithelium.² Stem cells, therefore, are of interest for their potential use in regenerative medicine.

Recent progress has identified potential molecular signatures of embryonic stem (ES) cells that delineate pathways that are used by somatic stem cells in the maintenance of self-renewal and in cell fate decisions.³ Among these markers of stemness, *Cripto-1* represents an important component of a critical core pathway that is used by ES cells. In this review we highlight the role of *Cripto-1* in stem cell self-renewal and differentiation with particular emphasis on the cross talk with other ES cell genes. Finally, re-expression of *Cripto-1* in human cancers and its contribution to malignant progression is discussed.

Supported by Intramural Research Program of the National Institutes of Health, National Cancer Institute.

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Accepted for publication April 14, 2010.

CME disclosure: None of the authors disclosed any relevant financial relationships.

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Figure 1. Schematic diagram of human Cripto-1 protein (amino acids 1-188). Cripto-1 is a GPI-anchored membrane protein that can be cleaved by GPI-PLD and released into the supernatant of the cells as a soluble protein. GPI-PLD: glycosylphosphatidylinositol phospholipase D.

Cripto-1, a Member of the Epidermal Growth Factor-Cripto-1-FRL-1-Cryptic Family, during Embryonic Development

The Epidermal Growth Factor-Cripto-1-FRL-1-Cryptic Gene Family and Protein Structure

Human Cripto-1 (also known as teratocarcinoma-derived growth factor-1) is the original member of the epidermal growth factor (EGF)-cripto-1-FRL-1-cryptic (CFC) family of vertebrate signaling molecules.⁴ Structurally, the EGF-CFC proteins contain a signal sequence for extracellular secretion, a modified EGF-like domain, a conserved cysteine-rich domain (CFC-motif), and a short hydrophobic carboxy-terminus, which contains sequences for glycosylphosphatidylinositol (GPI) attachment and cleavage (Figure 1). Removal of the GPI anchor by GPI-phospholipase D generates a soluble form of biologically active Cripto-1, which can therefore function both as a cell-membrane anchored protein or as a soluble ligand after GPI anchor removal (Figure 1).^{5,6}

Cripto-1/Nodal/Growth and Differentiation Factor-1/3 Signaling during Embryogenesis

Molecular genetic studies in mice, *Xenopus*, and Zebrafish have defined a functional link between EGF-CFC proteins and the transforming growth factor (TGF)- β superfamily member, Nodal. For instance, the EGF-CFC proteins function as co-receptors for Nodal and growth and differentiation factor (GDF)-1 and GDF-3 through a complex composed of Activin type II and type I (ALK4) serine threonine kinase receptors in the cell membrane.⁷ On Nodal/GDF-1/GDF-3 binding, the type I and type II receptor complex triggers phosphorylation of Smad-2 and Smad-3, which in turn bind to Smad-4 and, as a transcriptional complex, translocate to the nucleus to enhance transcription of specific target genes.⁷

During embryonic development, Cripto-1/Nodal signaling is involved in regulating the formation of the primitive streak, patterning of the anterior/posterior axis, specification of mesoderm and endoderm during gastrulation, and establishment of left/right (L/R) asymmetry of developing organs.^{4,8} Mouse embryos that lack the *Cripto-1* gene

(Cr-1^{-/-} mice) die at day 7.5 of embryogenesis due to defects in mesoderm formation and axial organization.⁹

Interestingly, Cr-1^{-/-} mice exhibit defects in myocardial development, as evidenced by the absence of expression of terminal myocardial differentiation genes.^{9,10} Genetic studies in humans have shown the involvement of Cripto-1 in the pathogenesis of ventricular septal defects, one of the most common congenital heart defects.¹¹ By using Cripto-1-deficient ES cells, insights into the functional role of Cripto-1 in cardiomyogenesis have been shown. In fact, while ES cells normally differentiate as embryoid bodies (EBs) into a wide variety of specialized cell types, including cardiomyocytes, Cr-1^{-/-} EBs do not form contracting cardiomyocytes.¹⁰ Notably, addition of Cripto-1 recombinant protein to Cr-1^{-/-} EBs during early phases of differentiation could effectively restore the ability of Cr-1^{-/-} EBs to differentiate into beating functional cardiomyocytes.¹²

Notably, Cr-1^{-/-} EBs spontaneously differentiate toward neurons, opening new possibilities for cell replacement therapy in neurodegenerative diseases such as Parkinson's disease. When wild-type ES cells are grafted at low concentrations into a mouse animal model of Parkinson's disease, they differentiate into midbrain dopaminergic neurons but they can also differentiate into other cell types, resulting in teratoma formation.¹³ In contrast, when Cr-1^{-/-} ES cells are grafted at low density, they generate tyrosine-hydroxylase positive cells that are able to restore the normal behavior in a Parkinson's disease animal model without producing teratomas.¹³

Cripto-1/Nodal Signaling as a "Signature" Signaling Pathway in ES Cells

The study of ES cell biology has produced an understanding of the transcriptional programming and molecular pathways that underlie stem cell self-renewal and differentiation. Such studies have identified molecular signatures of genes that control pluripotentiality and self-renewal in undifferentiated ES cells. Notably, *POU5F1* (*POU class 5 homeobox 1*)/*Oct-4*, *Nanog* and *Sox-2* were the first genes to be identified as key core modulators in maintaining an undifferentiated and pluripotential state in ES cells.¹⁴ These transcription factors regulate the expression of other transcription factors that either maintain or repress lineage specific differentiation genes. In 2007 the International Stem Cell Initiative characterized 59 human ES cell lines and found that all cell lines exhibited a similar expression profile for several markers of human ES cells, including the glycolipid stage specific embryonic antigens SSEA3 and SSEA4, the keratan sulfate antigens TRA-1-60, TRA-1-81, Nanog, Oct-4, GDF-3 and Cripto-1.³ Depletion of Oct-4 from human ES cells using RNA interference-mediated gene knockdown has identified Cripto-1 as a direct transcriptional target of Oct-4.¹⁵ Genome wide chromatin immunoprecipitation analysis has also shown the presence of Oct-4 and Nanog binding sites within the *Cripto-1* promoter region (Figure 2).¹⁶

Comparison of the transcriptional profile of both mouse and human ES cells has demonstrated that human and

Figure 2. Cross talk of the Nodal/Cripto-1 signaling pathway with *Wnt*, hypoxia, Notch, Oct-4, and Nanog signaling pathways. Activated Smad-2/Smad-3/Smad-4 complex can bind to specific TGF- β binding elements within the *Cripto-1* promoter and regulate *Cripto-1* gene expression. *Wnt* signaling pathway: *Cripto-1* is a downstream target gene of the *Wnt*/ β -catenin canonical pathway. Non-canonical *Wnt*11 can also bind to Cripto-1 and, in a complex with Frizzled 7 (Fzd7) and Glypican-4, induces β -catenin activation. Hypoxia: Hypoxia through HIF-1 α directly regulates *Cripto-1* expression in ES cells through binding to specific HREs within the *Cripto-1* promoter. ES cells specific transcription factors: Oct-4 and Nanog directly regulate *Cripto-1* expression in ES cells by binding to the *Cripto-1* promoter. Notch signaling pathway: Cripto-1 directly interacts with Notch in ER/Golgi membranes and enhances cleavage of the Notch extracellular domain, potentiating the Notch ligand-activated signal. Nodal-independent Cripto-1 signaling pathway: Cripto-1, independently of Nodal, binds to Glypican-1 and induces activation of c-Src/MAPK/AKT, leading to cell motility and EMT. TBE: TCF/LEF binding elements; HRE: hypoxia binding elements; SBE: Smad binding elements; CBP: CREB binding protein; TCF/LEF: T-cell factor/lymphoid enhancer factor. EMT indicates epithelial to mesenchymal transition; Notch ICD, Notch intracellular domain; ER, endoplasmic reticulum; GDF, Growth and Differentiation Factor; LRP, low-density lipoprotein receptor-related protein; and MAPK, mitogen activated protein kinase.

murine ES cells differ significantly on a global scale with transcript levels differences ranging from one- or twofold to fiftyfold. However, the distribution of some signature pathways is strikingly similar between murine and human ES cells, and genes such as *Oct-4*, *Lefty*, *Nodal*, *Sox-2*, *Utf-1* (undifferentiated embryonic cell transcription factor-1), *tert* (telomerase reverse transcriptase), and *Cripto-1* are highly enriched in both mouse and human ES cells.¹⁷ Therefore, these findings suggest that *Cripto-1* is a critical component of core pathways used by both mouse and human ES cells.

More recently, it has been reported that specific adult stem cells are capable of transdifferentiation. Remarkably, some pluripotency factors such as Oct4, Nanog, Sox-2, Klf4, and c-Myc have been shown to be involved in reprogramming differentiated cells into pluripotent ones. Mouse induced pluripotent stem cells were first reported in 2006 and human induced pluripotent stem cells in late 2007.^{18,19} *Cripto-1*, in conjunction with other pluripotency genes, was expressed by induced pluripotent stem cells derived from human keratinocytes, human

fibroblasts, and mouse liver and stomach cells.^{20–22} Given their distinctive regenerative abilities, stem cells offer the potential for treating diseases such as diabetes and heart disease. Moreover, these findings provide new insights into the fundamental mechanisms that regulate stem cell functions in normal tissues, and consequently lead to a better understanding of their deregulation during cancer.

Cross Talk of *Cripto-1* with Other Embryonic Genes

Several families of genes that are expressed by ES cells are also critical for early embryonic development. Among these genes are members of the *Wnt* signaling pathway, the transforming growth factor- β superfamily including *Nodal*, *Activin*, *GDF-1* and *-3*, the *Notch* pathway together with its signaling transcription factors, and *hypoxia-inducible factor (HIF)-1 α* . Notably, all these signaling pathways

have been shown to cross talk with Cripto-1, signaling suggesting a pivotal role played by Cripto-1 in the regulation of stem cell proliferation and differentiation (Figure 2).

Wnt Signaling Pathway

The canonical *Wnt*/ β -catenin signaling pathway has profound effects on cell function in mammals.²³ The key cytosolic transducer of the canonical *Wnt* pathway, β -catenin, is stabilized when cell surface receptors (LRP-5/6 and Frizzled family of proteins) are activated by secreted *Wnt* ligands. Stabilized β -catenin translocates to the nucleus where it interacts with the T-cell factor/lymphoid enhancer factor family of transcriptional activators, to activate target genes.²³ Several studies have shown that *Wnt* signaling and *Wnt* proteins are important for the maintenance of stem cells in the hair follicle of the skin, the crypt of the colon, and the hematopoietic and the nervous systems.²⁴ Activation of the *Wnt* signaling pathway has been shown to sustain the undifferentiated state of ES cells and to initiate and maintain carcinomas of the skin, intestine, liver, and brain.²⁴ However, *Wnt* signaling is only able to maintain pluripotent mouse ES cells in conjunction with the cytokine leukemia inhibitory factor (LIF), which following binding to its tyrosine kinase receptor gp130, activates and phosphorylates Stat3.²⁵ Interestingly, activation of the *Wnt* pathway can trigger transcription of genes that are also activated by stem cell transcription factors such as Oct-4, Nanog, and Sox-2.²⁶

Several studies have shown a potential interaction between the canonical *Wnt*/ β -catenin and the Nodal/Cripto-1 signaling pathways. In this regard, mouse and human *Cripto-1* have been identified as primary target genes in the *Wnt*/ β -catenin signaling pathway during embryonic development and in colon carcinoma cells (Figure 2).²⁷ Indeed, *Cripto-1* expression is down-regulated in β -catenin^{-/-} or Wnt3^{-/-} mutant mouse embryos. Likewise, *Wnt*/ β -catenin signaling also directly regulates *Nodal* in *Xenopus* and in the chick embryo during L/R axis determination.^{28,29} Reciprocally, phosphorylated Smad-2 protein, which is downstream of Nodal/Cripto-1/ALK4 signaling pathway, can activate the *Wnt*/ β -catenin signaling independently of Smad-4 through p300.³⁰ Finally, in *Xenopus*, the *Cripto-1* ortholog FRL-1 can function as a co-receptor for *Wnt11* in conjunction with the frizzled receptor Xfz7 and glypican-4, leading to activation and stabilization of β -catenin (Figure 2).³¹ This cross talk between the *Wnt*/ β -catenin and *Cripto-1* signaling pathways might be functionally significant, since activation of β -catenin in cells might lead to *Cripto-1* overexpression, thereby promoting cell proliferation and possibly transformation through a positive feed-forward loop.

TGF- β Family Members

The TGF- β signaling pathway performs important functions during embryonic development and organogenesis and during various stages of carcinoma formation in several different tissues.^{32,33} The TGF- β family includes the

TGF- β s, the bone morphogenetic proteins (BMPs), Activins, GDFs and Nodal, which are involved in embryonic developmental processes from the generation of organ asymmetries and correct positioning of the three main body axes to organ-specific morphogenesis.³² TGF- β family members can directly regulate *Cripto-1* expression in human embryonal carcinoma cells and in human colon cancer cells by binding to specific TGF- β binding elements within the *Cripto-1* promoter (Figure 2).³⁴

Nodal/Activin

In addition to performing a central role in the patterning of the early embryo during gastrulation, Nodal has been shown to maintain the pluripotency of human embryonic stem cells together with Activin and fibroblast growth factors (FGFs).³⁵ Recently, Vallier and colleagues³⁶ have demonstrated that Activin/Nodal signaling directly controls the expression of Nanog in human and mouse embryonic stem cells, blocking their differentiation toward neuroectoderm. A specific inhibitor of ALK4/5/7 type I receptors significantly decreases expression of pluripotency markers in embryonic stem cells and dramatically reduces their proliferation, indicating that Activin, Nodal, and other TGF- β molecules are essential for proliferation of mouse ES cells.^{37,38} In addition, recombinant Activin or Nodal proteins are able to stimulate proliferation of mouse ES cells without affecting their pluripotency, suggesting involvement of Activin/Nodal signaling in mouse ES cells self-renewal.^{37,38}

GDF-1 and GDF-3

Cripto-1, in addition to functioning as a co-receptor for Nodal, can also bind GDF-1 and GDF-3 (Figure 2).⁴ In the embryo, GDF-1 and GDF-3 are co-expressed with Nodal, suggesting a cooperative action among these ligands during embryonic development.³⁹ GDF-1 collaborates with Nodal during embryogenesis in establishing the L/R body asymmetry and both are required for transfer of the L/R asymmetric signal from the node to the lateral plate mesoderm.⁴⁰ GDF-3, in addition to functioning as a Nodal-like ligand, is also a direct bone morphogenetic protein inhibitor in early embryos and pluripotent stem cells.⁴¹ Recently, GDF-3 together with *Cripto-1* have been identified as ES cell markers that are obligatory and prominent for a population of ES cells, which are uncommitted and have high self-renewal capacity.⁴²

Lefty

Among the inhibitors of the Nodal signaling, Lefty and Cerberus are highly enriched in stem cells.^{43,44} Lefty antagonizes Nodal signaling by binding to *Cripto-1*, preventing Nodal from binding to its receptor complex.⁴ Furthermore, Lefty can also directly interact with Nodal in solution, preventing Nodal binding to *Cripto-1* and its receptor complex. For instance, secreted Lefty derived from human ES cells can down-regulate Nodal signaling in metastatic melanoma and breast carcinoma cells, reducing their tumorigenic phenotype *in vitro* and *in vivo*.⁴⁵

In addition to being abundant in undifferentiated ES cells, Lefty expression is also increased on differentiation of mouse ES cells after leukemia inhibitory factor withdrawal or retinoic acid treatment.⁴³ Therefore, the ability of Lefty to regulate stemness and differentiation in ES cells might be dependent on the regulation of Nodal and other TGF- β molecules that require Cripto-1 as a co-receptor for signaling.

The Notch Signaling Pathway

The Notch signaling pathway is critically important for normal development and stem cell fate decisions in several different tissues.⁴⁶ Genes of the *Notch* family encode four large transmembrane receptors (Notch1-4), which interact with membrane-bound ligands encoded by the Delta/Serrate/Jagged family genes (Delta-like1, Delta-like3, Delta-like4, Jagged1, and Jagged2). Under physiological conditions, the ligand expressed on one cell binds to a Notch receptor expressed on neighboring cells in a juxtacrine manner, thereby triggering a proteolytic cleavage of the intracellular domain of Notch (ICD) and its translocation into the nucleus. When the ICD enters the nucleus, it forms a complex with the DNA binding protein CSL (CBF-1, Suppressor of hairless, Lag-1, also known as RBP-Jk), activating transcription of downstream target genes such as *Hes-1* (*Hairy enhance of split-1*), *Hey*, *nuclear factor- κ B* (NF- κ B), *cyclin D1*, and *c-myc*.⁴⁶

During embryogenesis, Notch signaling favors maintenance of an undifferentiated state in stem and progenitor cells, regulating, for example, inhibition of neurogenesis and myogenesis. Involvement of Notch signaling has been also implicated in adult stem cells from the hematopoietic system, central nervous system, skin, and intestinal mucosa.⁴⁶ Furthermore, Notch signaling is frequently deregulated in human malignancies, with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, renal, pancreatic carcinomas, and lymphomas.⁴⁶

Mouse embryo mutants for Delta-like1 or double mutants for Notch1 and Notch2 exhibit multiple defects in L/R asymmetry, suggesting a cross talk of Notch signaling pathway with the Nodal/Cripto-1 signaling pathway.⁴⁶ Interestingly, analysis of the Node-specific enhancer within the mouse Nodal promoter has revealed the presence of binding sites for CSL/RBP-Jk protein, suggesting a direct regulation of Nodal expression by Notch signaling in L/R specification.^{47,48} Knockdown experiments using specific Notch small-interfering RNAs demonstrated that Notch4 might preferentially regulate Nodal expression increasing Nodal protein levels in malignant melanoma cells.⁴⁹ A recent report has also shown an additional novel interaction between the Notch and Cripto-1/Nodal signaling pathways.⁵⁰ In this study, through a yeast two-hybrid system and by co-immunoprecipitation analysis, Cripto-1 was found to directly bind to all four mammalian Notch receptors.⁵⁰ Binding of Cripto-1 to Notch1 could not be detected on the cell surface but occurred mainly in the endoplasmic reticulum/Golgi complex (Figure 2).

Similar to the regulation of Nodal processing by recruiting proprotein convertases to the Nodal precursor in early endosomes, Cripto-1 binding to Notch1 induces Notch1 localization in the lipid raft fraction of the endoplasmic reticulum and enhances cleavage of the Notch1 extracellular domain through a furin-like convertase. Enhanced cleavage of Notch-1 induced by Cripto-1 resulted in the sensitization to ligand-induced activation of Notch signaling in Chinese hamster ovary cells expressing a CBF1-dependent Notch reporter assay. Reciprocally, knockdown of Cripto-1 expression in mouse and human embryonal carcinoma cells desensitized ligand-induced Notch signaling activation. Therefore, enhancement of Notch signaling by Cripto-1 provides a new insight into the interaction between the Notch and Nodal/Cripto-1 signaling pathways and delineates a novel mechanism that might regulate embryogenesis and carcinogenesis.

Hypoxia and HIF-1 α

Hypoxia plays a central role in normal development, but also occurs in a variety of pathological conditions, including tumor growth, tissue ischemia, and inflammation.⁵¹ Cells respond to hypoxia through gene expression changes mediated by the HIF transcription factors. HIF is a heterodimeric transcription factor consisting of two subunits, an α subunit that is oxygen labile but is stabilized in response to low oxygen conditions and a β subunit also known as aryl hydrocarbon receptor nuclear translocator, which is constitutively expressed.⁵¹ Under low oxygen conditions, HIF-1 α is stabilized, dimerizes with HIF-1 β and translocates to the nucleus, where it binds to specific promoter elements known as hypoxia responsive elements (HREs) and activates target genes. Before the establishment of a vascular system, the early embryo develops in a naturally occurring hypoxic environment. Targeted disruption of HIF-1 α and aryl hydrocarbon receptor nuclear translocator in mice is embryonic lethal due mainly to cardiac and vascular defects, revealing the importance of HIF-mediated processes in embryonic development.⁵¹

Oxygen availability also regulates stem cell proliferation, survival, and differentiation. In fact, tissue stem cells reside within niches that are naturally hypoxic, and low oxygen levels regulate stem cell self-renewal and prevents their differentiation toward specific fates.⁵² *In vitro* studies using low oxygen culture conditions have also confirmed the direct influence of local oxygen concentration on stem cell self-renewal and pluripotency.⁵³ For instance, Ezashi and colleagues⁵⁴ have shown that human ES cells maintained under hypoxic conditions (1 to 4% oxygen) had a fully pluripotent state as compared with human ES cells growing in normoxic conditions (20% oxygen).

Although not affecting gene expression of *Oct4*, *Nanog*, and *Sox2* in human ES cells, hypoxic cultures led to transcriptional differences, including downstream targets of Oct4, Nanog, and Sox2, in human ES cells when compared with cultures growing in normoxic conditions.⁵⁵ Interest-

ingly, Lefty2, a TGF- β family member that plays a role in preventing spontaneous differentiation of human ES cells by blocking Nodal signaling, is down-regulated under normoxic conditions as compared with cultures in hypoxic conditions.⁵⁵ Lefty2 down-regulation at higher oxygen concentrations may render pluripotent human ES cells less stable and therefore more responsive to signals that drive differentiation. In contrast, low oxygen has the opposite effect on mouse ES cells, reducing the expression of pluripotency genes, although other reports have provided conflicting results.⁵³ A low oxygen microenvironment can also influence differentiation during development. In fact, several studies have reported that differentiation under low oxygen increases the generation of neurons, cardiomyocytes, hemangioblasts, hematopoietic progenitors, endothelial cells and chondrocytes.⁵³

In addition, cross talk between HIFs and key stem cell regulatory pathways has been discovered. HIF-2 α , but not HIF-1 α , is a direct upstream regulator of Oct-4 and binds to specific HREs within the promoter of murine *Oct-4*.^{51,56} HIF-1 α can also directly interact with β -catenin and with Notch1.⁵⁶ A first clue of the link between Notch and hypoxia derives from studies demonstrating that the Notch target gene *Hes1* was up-regulated by hypoxia in neuroblastoma cell lines.⁵⁷ In addition, Gustafsson and collaborators⁵⁸ demonstrated that hypoxic response required functional Notch signaling to maintain an undifferentiated cell state. At the molecular level, HIF-1 α interacts with Notch1 ICD and is recruited to DNA regions containing CSL-binding sites. HIF-1 α becomes part of the Notch ICD/CSL transcriptional complex, increasing expression of Notch downstream target genes *Hes-1* and *Hey-2*.⁵⁶ Some of these genes contain both HRE- and CSL-binding sites, suggesting that hypoxia and Notch may have additive effects on transcription.⁵⁹ Therefore, a hypoxic niche may be particularly favorable for the optimal activation of Notch targets that promote cell quiescence and inhibit cell differentiation.

Hypoxia through HIF-1 α can also directly regulate Cripto-1 expression by binding to HREs within the promoter of the mouse and human *Cripto-1* genes in mouse ES cells and in human embryonal carcinoma cells (Figure 2).⁶⁰ Furthermore, hypoxia can modulate the differentiation of mouse ES cells and can enhance formation of beating cardiomyocytes as compared with mouse ES cells that are differentiated under normoxic oxygen conditions. However, hypoxia failed to induce differentiation of mouse ES cells into cardiomyocytes in the absence of Cripto-1 expression,

suggesting that Cripto-1 is required for hypoxia to fully differentiate mouse ES into cardiomyocytes.⁶⁰ Therefore, Cripto-1 might represent an additional HIF-1 α target gene in the hypoxic niche and, together with other genes such as *Oct-4*, *Notch*, and *Wnt*, might regulate stem cell proliferation and self-renewal.

Cripto-1 in Human Cancer and as a Marker of Cancer Stem-Like Cells

Cripto-1 Oncogenic Activities in Vitro and in Vivo

Several studies have demonstrated that Cripto-1 induces *in vitro* transformation of normal epithelial cells, promotes epithelial-to-mesenchymal transition (EMT), and stimulates angiogenesis (Table 1).⁴ Stimulation of cell proliferation, motility, survival, and EMT by Cripto-1 is independent of Nodal and relies on activation of the c-Src/mitogen activated protein kinase/AKT signaling pathways through binding of Cripto-1 to the heparan sulfate proteoglycan Glypican-1 (Figure 2).⁴

Transgenic mouse models have shown that overexpression of a human Cripto-1 transgene in the mouse mammary gland under the control of the mouse mammary tumor virus or whey acidic protein promoter results in mammary hyperplasias and adenocarcinomas (Table 1).^{61,62} Interestingly, mammary tumors of mixed histology with regions containing glandular, papillary, myoepithelioma, adenosquamous, and undifferentiated carcinoma were identified in multiparous whey acidic protein Cripto-1 female mice.⁶² Mammary tumors of mixed histology are normally phenotypes that are associated with transgenic mice that have alterations in the canonical Wnt/ β -catenin pathway. In fact, increased expression of an activated β -catenin was found in mammary tumors of whey acidic protein-Cripto-1 transgenic mice, suggesting that a canonical Wnt pathway may be activated in these tumors.⁶²

In addition, Cripto-1 is expressed at high levels in different types of human tumors, including breast, colon, gastric, pancreatic, lung, cervical, endometrial, skin, testis, bladder, and ovarian carcinomas.⁴ Therefore, *Cripto-1* might represent an example of an embryonic gene that is re-expressed, possibly in stem cells in adult tissues, in an inappropriate fashion and thereby may contribute to the pathogenesis of cancer. Clearly, there are other embryonic genes that regulate stem cell function, such as those belonging to the

Table 1. Cripto-1 Oncogenic Activities

<i>In vitro</i>	<i>In vivo</i>
Stimulation of proliferation in serum-free conditions	Mammary hyperplasias and adenocarcinomas in transgenic mouse models
Stimulation of colony formation in semi-solid medium (agar)	EMT in transgenic mouse models
Enhanced motility and invasion in Boyden chamber assay	Inhibition of milk proteins production during pregnancy
Enhanced branching morphogenesis in type I collagen gel and Matrigel	Increased blood vessel formation in a xenograft tumor model
Induction of mesenchymal markers (Snail, slug, vimentin, N-cadherin) and loss of epithelial adhesion molecules (E-cadherin) (EMT)	Formation of new blood vessels in directed <i>in vivo</i> angiogenic assay (DIVAA)
Stimulation of microvessel formation in Matrigel	

TGF- β , *Notch*, *Wnt* and *Hedgehog* families, which are also overexpressed in human tumors, confirming this connection between stem cells and cancer.⁴

Cripto-1 and Cancer Stem Cells

It has been suggested that the capacity of a tumor to grow is due to a small subset of cells within the tumor, called cancer stem cells or tumor initiating cells.⁶³ Cancer stem cells have been identified and isolated from tumors of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck, and pancreas. Cancer stem cells are able to self-renew and regenerate the original phenotype of the tumor when implanted into immunodeficient mice.⁶³

Emerging evidence clearly suggests that cancers can grow and metastasize from cancer stem cells, and recent data has shown that Cripto-1 is highly expressed in a subpopulation of human embryonal carcinoma cells with cancer stem-like characteristics.⁶⁴ We have in fact found that the expression of Cripto-1 in embryonal carcinoma cells is heterogeneous with two distinct subpopulations of Cripto-1 high and Cripto-1 low expressing cells. These two subpopulations show different *in vitro* and *in vivo* tumorigenic capacities. The Cripto-1 high subpopulation showed a higher ability than the Cripto-1 low subpopulation to form tumor spheres in serum-free suspension culture. In addition, embryonal carcinoma cells that were injected subcutaneously into nude mice from Cripto-1 high-expressing cells formed tumors that were larger and had a shorter tumor latency period compared with tumors that arose from Cripto-1-low expressing cells. However, Cripto-1 high-expressing cells could be generated from Cripto-1 low-expressing cells over a certain period of time both *in vitro* and *in vivo*, suggesting that plasticity of embryonal carcinoma cells might be responsible of the reversible phenotype between Cripto-1 high and Cripto-1 low populations. Finally, key regulators of pluripotent ES cells, such as Activin/Nodal and the transcription factors Nanog and Oct-4, modulated Cripto-1 expression in this subpopulation of embryonal carcinoma cells. In addition to embryonal carcinomas, Cripto-1 has also been identified in a small subset of stem-like cells in human malignant melanomas and in androgen-responsive and refractory human prostate tumor cell lines.^{65,66}

Cripto-1, EMT, and Cancer Stem Cells

Reports from several laboratories have suggested a link between stem cells and cells that have undergone EMT.⁶⁷ Indeed, normal and malignant breast stem-like cells were found to express markers of EMT.⁶⁸ Moreover, induction of EMT in immortalized human mammary epithelial cells resulted in the expression of stem cell markers and in an increased ability to form mammospheres *in vitro*, suggesting that EMT performs an important role in generating cancer stem-like cells in human breast tumors.⁶⁸ Since Cripto-1 has been found to promote EMT *in vitro* in mouse mammary epithelial cells and *in vivo* in mouse mammary tumors, it is likely that Cripto-1 might be associated with the EMT gene expression program of breast cancer stem-like cells, sup-

porting their self-renewal, invasive, and metastatic abilities.⁶⁹

Conclusions

In conclusion, the Nodal/Cripto-1 signaling pathway may represent an attractive target for treatment in cancer because Cripto-1 targeting will not only eliminate differentiated cancer cells but also should destroy an undifferentiated subpopulation of tumor cells that exhibit mesenchymal and stem-like characteristics, thereby leading to eradication of the tumor. However, it is imperative to design new strategies targeting the inactivation of Cripto-1 signaling based on the molecular understanding of the cross talk between Cripto-1 and other signaling pathways that control the biology of self-renewal and survival capacity of tumor initiating cells.

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