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Targeting the embryonic gene Cripto-1 in cancer and beyond

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Abstract

Importance of the field—Emerging evidence has clearly implicated an inappropriate activation of embryonic regulatory genes during cell transformation in adult tissues. An example of such a case is the embryonic gene Cripto-1. Cripto-1 is critical for embryonic development and is considered a marker of undifferentiated embryonic stem cells. Cripto-1 is expressed at low levels in adult tissues, but is re-expressed at a high frequency in a number of different types of human carcinomas, therefore representing an attractive therapeutic target in cancer.

Area covered in this review—This review surveys different approaches that have been used to target Cripto-1 in cancer as reflected by the relevant patent literature as well as peer-reviewed publications. Potential involvement and targeting of Cripto-1 in neurodegenerative and degenerative muscle diseases are also discussed.

What the reader will gain—The reader will gain an overview of different monoclonal antibodies, vaccines or oligonucleotides antisense targeting Cripto-1. A humanized anti-Cripto-1 antibody is currently being tested in a phase I clinical trial in cancer patients.

Take home message—Targeting Cripto-1 in human tumors has the potential to eliminate not only differentiated cancer cells but also destroy an undifferentiated subpopulation of cancer cells with stem-like characteristics that support tumor initiation and self-renewal.

1. Introduction

1.1 Human Cripto-1, a member of the EGF-CFC gene family

Human Cripto-1 is a cell membrane-anchored protein that has been shown to play an important role in embryonic development and in tumor progression [1,2]. Cripto-1 belongs to the Epidermal Growth Factor/Cripto/FRL-1/Cryptic (EGF-CFC) gene family [1,2]. EGF-CFC family genes share well conserved structural modules such as intron-exon organization, suggesting that these genes are evolutionally related and they probably derived from a common ancestor gene [3,4]. Although the overall primary sequence identity is low (22–32%), EGF-CFC family members exhibit a unique and highly conserved structural profile containing a NH₂-terminal signal peptide, a variant EGF-like domain, a Cripto-FRL-1-Cryptic (CFC) motif and a short hydrophobic COOH-terminal segment, which functions as glycosylphosphatidylinositol (GPI) cleavage and attachment signal [1,2]. In addition to its primary structure, Cripto-1 is processed post-translationally as a GPI-anchored glycoprotein. Biochemical characterization by peptide mapping, mass spectrometric analysis, and glycosidase treatment of a COOH-deleted soluble form of Cripto-1 protein revealed several

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glycosyl modification sites, including O-linked glycosylation at Ser40 and Ser161 (which is the ω site for GPI-attachment), N-linked glycosylation at Asn79, and O-linked fucosylation at Thr88 [5–9]. Among them, the O-linked fucose modification is rare and exclusively found within the EGF-like domain of extracellular proteins, such as urinary-type plasminogen activator (uPA), coagulation factors VII and IX, and Notch receptors [10,11]. O-linked fucosylation of EGF-CFC proteins has been shown to be necessary for activity of human and mouse Cripto-1 proteins in a Nodal-dependent signaling pathway, although another study has demonstrated that is the Thr88 residue and not fucosylation of this residue that is required for Cripto-1 to function as a Nodal co-receptor [8,9]. For instance, mutation of the threonine residue to alanine completely abrogated activity of Cripto-1 protein with respect to induction of a Nodal-dependent signaling pathway [8,9]. However, Cripto-1 O-fucosylation mutants are fully functional with regard to activation of Nodal-independent signaling pathways [12]. Another important post-translational modification in EGF-CFC proteins is the GPI-modification. GPI-anchoring determines membrane localization of Cripto-1 in lipid rafts microdomains and within caveolae [13]. The Cripto-1 protein can be released from the cell membrane following treatment with phosphatidylinositol-phospholipase C (PI-PLC), and by the activity of the endogenous enzyme GPI-phospholipase D (GPI-PLD) [5]. Therefore, this controlled release mechanism may define the activity of Cripto-1 as a membrane-associated co-receptor or a soluble ligand. In fact, soluble forms of Cripto-1 have been reported to be active in a number of different *in vitro* and *in vivo* assays, while the GPI-anchor is required by Cripto-1 to function as a co-receptor for Nodal [6].

2. Intracellular signaling pathways activated by Cripto-1

2.1 Cripto-1/Nodal-dependent signaling pathway during embryonic development

Cell-membrane attached Cripto-1 functions as a co-receptor with the type I Activin serine-threonine kinase receptors, Alk4 or Alk7, for the transforming growth factor β (TGF- β)-related peptides Nodal and Growth and Differentiation factor 1 and 3 (GDF1 and GDF3) [14,15]. Nodal and Cripto-1 are inactive independently and together induce activation of an Activin type II (ActRIIA or ActRIIB) and type I receptor complex. Activation of Alk4 can in turn phosphorylate Smad-2 and Smad-3, which in turn bind to Smad-4 and translocate to the nucleus enhancing transcription of specific target genes [14,15]. While Nodal signaling through Alk4 is fully dependent upon interaction with Cripto-1, Nodal can bind directly to Alk7 signaling in the absence of Cripto-1 [16]. However, Cripto-1 is still able to significantly potentiate the responsiveness of the Alk7/ActRIIB complex to Nodal, indicating that both Alk7 and Alk4 cooperate together with Cripto-1 in modulating Nodal signaling [16]. Therefore, a critical function of Cripto-1 during embryonic development is to mediate Nodal/GDF1/GDF3 signaling through Alk4 or Alk7 receptors. In addition, Nodal signaling during embryogenesis can be modulated by several antagonists. Some of these inhibitors (i.e. Tomoregulin-1, lefty and antivin) have been shown to antagonize Nodal signaling by directly interacting with Cripto-1 and competing with Nodal/GDF1 for binding to Cripto-1 [1,14]. Cripto-1 can also act as an inhibitor of Activin and TGF- β 1 signaling. For instance, Cripto-1 can directly bind to Activin A, Activin B or TGF- β 1 disrupting the ability of these signaling molecules to bind and activate a functional type I/type II receptor complex [17–19]. Since Activins and TGF- β 1 are potent inhibitors of cell growth in different cell lines, antagonism of Activin/TGF- β 1 signaling might represent one of the mechanisms by which Cripto-1 regulates and promotes tumorigenesis. Furthermore, the endoplasmic reticulum chaperone Glucose Regulated Protein 78 (GRP78) can directly bind Cripto-1 and the Cripto-1/GRP78 protein complex can antagonizes TGF- β signaling in prostate cancer cells, leading to the inhibition of TGF- β mediated growth arrest under anchorage-dependent and independent conditions [20].

2.2 Cripto-1/Nodal-independent signaling pathway during oncogenic transformation

In addition to functioning as a co-receptor for Nodal/GDF1/GDF3, Cripto-1 can signal as a ligand for Glypican-1, a GPI-anchored heparan sulphate proteoglycan (HSPG) that is tethered to the plasma membrane in lipid rafts [21]. Binding of Cripto-1 to Glypican-1 leads to phosphorylation of the cytoplasmic tyrosine kinase c-src. Activation of c-src in turn activates mitogen-activated protein kinase (MAPK)/ phosphatidylinositol 3' kinase (PI3K)/ Akt signaling pathways that regulate cell proliferation, motility and survival [14,21]. Activation of the MAPK and PI3K/Akt signaling pathways is independent of Nodal and Alk4, since Cripto-1 can enhance phosphorylation of MAPK and Akt in cell lines lacking Alk4 and/or Nodal expression [22]. The tyrosine kinase c-src is required by Cripto-1 to induce *in vitro* transformation of mouse mammary epithelial cells, indicating that inappropriate activation of c-src by Cripto-1 in a Nodal- and Alk4-independent manner might play a key role in Cripto-1 tumorigenic activity [21]. GRP78 has also been shown to be essential for the activation of the MAPK/Akt signaling pathway by Cripto-1 [20]. For instance, blockade of the Cripto-1/GRP78 interaction strongly interferes with the oncogenic activity of Cripto-1 *in vitro* by blocking the activation of the MAPK/Akt signaling pathway. Finally, cross-talk of Cripto-1 with the *wnt*/β-catenin and Notch signaling pathways has been reported [23–25]. Since the *wnt*/β-catenin and Notch signaling pathways are frequently deregulated in cancer, crosstalk of Cripto-1 with these signaling pathways might be functionally significant, by enhancing Cripto-1 oncogenic activities *in vitro* and *in vivo*.

3. Cripto-1 oncogenic activities *in vitro*

Several studies have clearly demonstrated that expression of Cripto-1 at high levels in a variety of normal cell lines enhances their ability to grow in serum-free conditions and to form colonies in soft agar, inducing a transformed phenotype *in vitro* [26–28]. Overexpression of Cripto-1 in mouse mammary epithelial cells and in breast or cervical carcinoma cells is also able to increase their motility, as assessed by Boyden chamber migration and invasion assays [29–31]. Interestingly, biochemical changes that characterize epithelial to mesenchymal transition (EMT) were observed in HC-11 mouse mammary epithelial cells overexpressing Cripto-1 [32]. For instance, the intercellular adhesion molecule E-cadherin was significantly decreased in HC-11 Cripto-1 expressing cells. In contrast, HC-11 Cripto-1 cells showed a significant increase in the expression of N-cadherin, vimentin, the zinc-finger repressor transcription factor snail, as well as an increase in the phosphorylated forms of signaling molecules such as c-src, focal adhesion kinase (FAK) and Akt, which are known to be activated during EMT. Cripto-1 can also enhance proliferation, migration, invasion and formation of vascular structures in Matrigel of human umbilical endothelial cells (HUVECs), indicating that Cripto-1 might function as an angiogenic molecule [33]. Furthermore, enhanced tumor vascularization was observed in MCF-7 Cripto-1 overexpressing tumor xenografts in nude mice and elevated microvessel formation *in vivo* was detected by a directed *in vivo* angiogenic assay (DIVAA) [33]. Since Cripto-1 expression can be directly regulated by hypoxia-inducible factor-1 α (HIF-1 α), it is possible that hypoxic conditions, that are often found within growing tumors, might trigger Cripto-1 expression leading to enhanced tumor vascularization [34].

4. Cripto-1 oncogenic activities *in vivo*

Transgenic mouse models have clearly implicated Cripto-1 in cellular transformation *in vivo*. Overexpression of a human Cripto-1 transgene in the mouse mammary gland under the control of the mouse mammary tumor virus (MMTV) or the whey acidic promoter (WAP) results in enhanced mammary ductal branching, intraductal hyperplasia and hyperplastic alveolar nodules in virgin mice [35,36]. Approximately 30–50% of multiparous Cripto-1 transgenic female mice develop mammary tumors. While the mammary tumors in the

MMTV-Cripto-1 transgenic mice are predominantly papillary adenocarcinomas, the mammary tumors found in the WAP-Cripto-1 transgenic mice, in addition to papillary adenocarcinomas, also contain areas of microglandular, myoepithelial and adenosquamous carcinomas [35,36]. Mammary tumors with mixed histotypes are typical of MMTV-*wnt-1* transgenic mice and increased expression of activated β -catenin was indeed detected in mammary tumors of WAP-Cripto-1 transgenic mice, suggesting that the canonical β -catenin/*wnt* pathway might play a role during mammary transformation induced by Cripto-1 *in vivo* [37,38].

5. Cripto-1 expression in human tumors

Expression of Cripto-1 mRNA and/or protein have been detected in a variety of human tumors, including colorectal, breast, gastric, pancreatic, ovarian and lung carcinomas [14,15]. While Cripto-1 is usually expressed at low levels in normal adult tissues, a significant increase in Cripto-1 expression has been shown in premalignant lesions of the colon, stomach and breast [39–42]. Cripto-1 expression has also been detected in patients with long-term *Helicobacter pylori* infection and atrophic gastritis, two risk factors for gastric carcinogenesis, and in the normal colon mucosa from individuals that belong to families with high risk of colorectal carcinomas [43,44]. Recently, high levels of Cripto-1 expression in colorectal tumors have been found to be associated with metachronous metastasis and poor prognosis [45]. Cripto-1 overexpression is also a prognostic factor in breast cancer patients. According to one study, Cripto-1 is expressed at high levels in approximately 47.5% of breast cancer patients, as assessed by immunohistochemical analysis [46]. Univariate analysis revealed a significant correlation between Cripto-1 expression and poor prognosis, while multivariate analysis confirmed that Cripto-1 expression is an independent prognostic factor in breast cancer patients. In addition to be expressed in breast and colon cancer tissues, soluble Cripto-1 can also be detected in the plasma of colon and breast cancer patients. Using a highly sensitive and specific sandwich-type enzyme-linked immunosorbent assay (ELISA) for Cripto-1, a statistically significant increase in the plasma levels of Cripto-1 was found in colon and breast cancer patients when compared with a control group of healthy volunteers [47]. Elevated Cripto-1 plasma levels were also detected in breast cancer patients at an early stage, indicating that Cripto-1 might be useful in the early diagnosis of this disease. No significant correlation between Cripto-1 levels in the plasma of colon and breast cancer patients and various clinicopathologic parameters was found, probably due to the small sample size analyzed in this study [47].

6. Targeting Cripto-1 in human carcinomas

6.1 Antisense oligonucleotides

Since Cripto-1 is expressed at high levels in human carcinomas, as compared to normal tissues, Cripto-1 represents a potential target for therapeutic intervention in cancer. Different therapeutic approaches have been used to block Cripto-1 activity and/or expression, such as antisense (AS) oligonucleotides that reduce Cripto-1 expression or neutralizing monoclonal antibodies that block the activity of the Cripto-1 protein [19,48,49]. Sequence specific AS oligonucleotides or AS expression vectors have been successfully used to impair Cripto-1 expression in several different types of human carcinoma cells [50,51]. For instance, treatment of breast, ovarian and colon cancer cell lines with Cripto-1 AS oligonucleotides resulted in a significant growth inhibition *in vitro* [51–54]. Interestingly, an additive effect was observed on reducing the growth of breast, ovarian and colon cancer cells when a Cripto-1 AS oligonucleotide was combined with AS oligonucleotides directed against transforming growth factor (TGF)- α and amphiregulin (AR), suggesting that different growth factors cooperate to regulate proliferation of cancer cells [51–54]. Furthermore, an additive or synergistic growth inhibitory effect was observed when colon cancer cells were

treated by a Cripto-1 AS oligonucleotide in combination with conventional chemotherapeutic agents, or with an anti-human EGF receptor monoclonal antibody C225 or with a 8-Cl-cAMP analog, that specifically inhibits type I protein kinase A [48,55,56]. Efficacy of Cripto-1 AS oligonucleotides, alone or in combination with TGF- α AS and/or AR AS oligonucleotides, has also been demonstrated *in vivo*. In fact, TGF- α , AR and CR-1 AS oligonucleotides were able to inhibit the growth of colon cancer tumor xenografts in nude mice in a dose dependent manner, while a more significant tumor growth inhibition was observed when mice were treated with a combination of the three AS oligonucleotides [52].

6.2 Monoclonal antibodies

Monoclonal antibodies that recognize antigens on the surface of tumor cells are effective therapeutic agents that are currently used to treat a variety of diseases [57]. Because Cripto-1 is a cell surface protein overexpressed by a variety of different carcinoma cells tumor cells with very little to no expression in normal adult tissues, targeting Cripto-1 in cancer patients might be promising. Mouse monoclonal antibodies that bind Cripto-1 have been previously described [19,49]. Adkins and colleagues of Biogen-Idex have generated a panel of monoclonal antibodies directed against a human recombinant Cripto-1 protein expressed as a human IgG1 Fc fusion protein (Fig. 1) [19]. Based on their ability to recognize different domains of the Cripto-1 protein in an ELISA assay, the anti-Cripto-1 antibodies have been classified in four classes: N-terminal tip, N-terminal, EGF domain and CFC domain. The anti-Cripto-1 monoclonal antibodies were able to recognize by immunohistochemical and immunofluorescent staining Cripto-1 expression in human breast and colon tumor tissue samples and in human cancer cell lines. N-terminal monoclonal antibodies, including B3.F6.1 and A10.B2.18, showed binding activity to a cell surface Cripto-1 protein expressed in tumor tissues or cell lines with no inhibition of Cripto-1 signaling functions. In contrast, the anti-EGF domain monoclonal antibodies, such as A27.F6.1 or A8.H3.1, were able to block interaction of Cripto-1 with Nodal and therefore inhibit Cripto-1-dependent Nodal signaling through the Smad-2/-3 pathway in NCCIT embryonal carcinoma cells. Anti-CFC monoclonal antibodies, such as A8.G3.5, also efficiently blocked Nodal signaling in NCCIT, by disrupting Cripto-1 interaction with Alk4. Surprisingly, anti-CFC monoclonal antibodies, in addition to interfering with the interaction of Cripto-1 with Alk4, also blocked interaction of Cripto-1 with Activin B [19]. Since overexpression of Cripto-1 can antagonize the Activin B growth inhibitory activity in breast cancer cells, addition of anti-CFC monoclonal antibodies were found to reverse the Cripto-1 inhibition of Activin B signaling and thereby restored the growth inhibitory function of Activin B in tumor cells. Monoclonal antibodies targeting either the EGF or the CFC domains of Cripto-1 inhibited tumor growth of NCCIT and GEO cells that were grown subcutaneously in nude mice. However, A8.G3.5 anti-CFC monoclonal antibody showed a more potent inhibition of tumor growth *in vivo* as compared to anti-EGF-monoclonal antibodies. In contrast, the N-terminal monoclonal antibodies, including B3.F6.1, showed no significant effects on the tumor growth of NCCIT or GEO xenografts, indicating that monoclonal antibodies targeting the N-terminal region of Cripto-1 are only binding antibodies without any blocking activity. Interestingly, Biogen-Idex has chosen not to use a Cripto-1 blocking antibody, such as the anti-CFC monoclonal antibody A8.G3.5, but has selected to use an N-terminal binding antibody, such as B3.F6.1, to target Cripto-1 in human tumors, probably because of the higher binding affinity and range of reactivity of this antibody with tumor-derived Cripto-1 (US0008906) [58]. Since murine antibodies can be immunogenic when administered in humans and since they can generate a neutralizing antibody response (human anti-murine antibody (HAMA) response) when they are administered repeatedly in chronic diseases such as cancer, Biogen-Idex describes in their patent "Cripto-1 binding molecules" various humanized forms of the N-terminal B3.F6.1 monoclonal antibody [58,59]. Since the B3.F6.1 monoclonal antibody lacks of any significant blocking activity, they conjugated the antibody to a cytotoxin.

Maytansinoids are plant derived anti-fungal and anti-tumor agents that have shown anti-leukemic effects in murine models [60]. However, maytansinoids have an unacceptable toxicity, including central and peripheral neuropathies [60]. In contrast to the high cytotoxicity of free maytansinoids, an antibody conjugated with maytansinoid has a toxicity that is much lower on antigen-negative cells as compared to antigen-positive cells. The maytansinoid linked to B3-F6-1 antibody is a C3 ester of maytansinol (DM4). Upon internalization by cells expressing Cripto-1, the DM4 moiety binds to tubulin, disrupting microtubule structures and leading to inhibition of cell division [58]. Biogen-Idec has also generated humanized full-length antibodies and humanized antibodies lacking the CH2 domains [58]. CH2 deleted antibodies are dimeric binding molecules containing two different isoforms: Form A, comprising two heavy chain portions linked via an inter-chain disulfide linkage, and Form B, where the two heavy chains are not linked by a disulfide bond. Since Form A shows enhanced stability *in vitro* and enhanced biodistribution *in vivo*, it was preferable to use and produce Form A over Form B. This was achieved by inclusion of synthetic connecting peptides that result in the preferential biosynthesis of Form A over Form B. The connecting peptide G1/G3/Pro243Ala244Pro+[Gly/Ser] hinge described in this patent resulted in the production of all Form A of a CH2 deleted chimeric B3.F3.1 antibody (chB3.F6.1ΔCH20), an anti-Cripto-1 monoclonal antibody consisting of murine heavy and light chain variable domains fused to human heavy and light chain constant domains [58]. Binding of chB3.F6.1ΔCH20 antibody containing the connecting peptide and control chB3.F6.1 IgG antibody was evaluated in flow cytometry assays on GEO colon cancer cells. Binding of the chB3.F6.1ΔCH20 antibody to GEO cells was indistinguishable from the control chB3.F6.1 antibodies, indicating that the chB3.F6.1ΔCH20 antibody still retains the full binding activity of the control parental antibodies. Next, they evaluated the anticancer activity of the full length anti-Cripto-1 humanized antibodies *in vivo* in nude mice [58]. Investigators at Biogen-Idec implanted subcutaneously solid tumor fragments in nude mice from a serially passaged *in vivo* breast cancer donor line (BT-474), or NCCIT human embryonal carcinoma cells or CLAU-6 human lung carcinoma cells. Two weeks after implantation, mice were randomized into control or treatment groups. In the BT-474 breast cancer xenograft model, humanized B3.F6.1 antibody conjugated to DM4 at 35 mg/kg/injection resulted in a statistically significant inhibition of tumor growth from day 29 to day 59, when the vehicle control group was terminated. Humanized B3.F6.1-DM4 antibody also produced a regression of established NCCIT human embryonal carcinoma xenografts at 10, 15 and 25 mg/kg/injection beginning on day 20. Tumors remained regressed until the end of the study on day 107, indicating that humanized B3.F6.1-DM4 antibody is highly effective in inhibiting tumor growth of NCCIT xenografts. Humanized B3.F6.1-DM4 antibody was also able to significantly inhibit the growth of CLAU-6 lung cancer xenografts at 10 and 20 mg/kg/injection. A significant growth inhibition was also detected in tumor xenografts in mice treated with a CH2 deleted humanized anti Cripto-1 antibody. Several mice treated with the highest concentrations of humanized B3.F6.1-DM4 antibody were diagnosed with coryneform bacterial infections of the skin, which is considered an opportunistic infection that occurs when the animal is stressed. The humanized anti-Cripto-1-DM4 conjugated monoclonal antibody is currently being evaluated in phase I clinical trials for treatment of patients with refractory or relapsed Cripto-1 positive solid tumors (A phase I study of BIIB015 in relapsed/refractory solid tumors, protocol ID 207ST101/NCT00674947). Another group at the Austin Research Institute in Heidelberg, Australia, has also developed rat monoclonal antibodies directed against a 17 amino acid peptide sequence corresponding to residues 97–113 of the human Cripto-1 protein that is part of the EGF-like domain of Cripto-1 (US0119514) (Fig. 1) [61,62]. Three IgM monoclonal antibodies (C3, C4 and C13) were characterized and they showed strong reactivity by immunostichemical analysis, fluorescent staining or western blot analysis with native Cripto-1 protein expressed by tumor tissues or cancer cell lines. Anti Cripto-1 rat monoclonal antibodies did not react with normal tissues or with human embryonal kidney 293 cells, which are negative for Cripto-1

expression. Furthermore, anti-Cripto-1 rat monoclonal antibodies showed a significant anti-proliferative effect *in vitro*, inhibiting specifically the growth of human breast and colon cancer cells, as assessed by ³H-thymidine incorporation. Anti-Cripto-1 rat monoclonal antibodies were also able to increase the sensitivity of LS174T human colon cancer cells and MCF-7 human breast cancer cells to the cytotoxic effects of drugs such as cisplatin, 5-Fluorouracil, carboplatin and epirubicin [61]. C13 monoclonal antibody could also inhibit cancer cell growth *in vivo* in LS174T xenografts in SCID mice, inducing up to 80% growth inhibition of established tumors as compared to an untreated control group. Interestingly, C4 and C13 rat monoclonal antibodies were highly effective in reducing tumor growth of a human leukemia multidrug resistant (MDR) cell line (CEM/A7R) both *in vitro* and *in vivo* in an established xenograft tumor model [63]. Additionally, C4 and C13 monoclonal antibodies significantly enhanced sensitivity of CEM/A7R MDR resistant leukemia cells to chemotherapeutic agents, such as epirubicin, daunorubicin and cytosine arabinoside [63]. They also demonstrated that the mechanism by which these anti-Cripto-1 rat monoclonal antibodies induced inhibition of tumor cell growth was through the activation of apoptosis. In fact, anti-Cripto-1 rat monoclonal antibody treated cells showed DNA fragmentation and cleavage of poly (ADP-ribose) polymerase (PARP), indicating activation of caspases [61,63]. In addition, the anti-Cripto-1 rat monoclonal antibodies inhibited the survival pathway Akt and induced activation of the proapoptotic pathways c-Jun-N-terminal kinase (JNK) and p38 MAPK [49]. Peter Gray and Wylie Vale of the Salk Institute for Biological Studies in La Jolla, California, have also proposed the use of monoclonal antibodies to inhibit the interaction of Cripto-1 with GRP78 (US0135904) [64]. GRP78 is a stress response protein which normally resides in the ER, where it regulates protein folding and maturation, but can be expressed on the surface of tumor cells [65,66]. Cripto-1 through the CFC-domain directly interacts with GRP78 at the cell surface of several cancer cell lines and Cripto-1/GRP78 binding is independent of prior association in the ER [20]. Gray and colleagues also have demonstrated that GRP78 and Cripto-1 cooperate to enhance cell growth and inhibit the growth inhibitory effect of TGF- β and Activins in human prostate cancer cells [20]. Furthermore, GRP78 amplified the Cripto-1-dependent activation of the c-src/MAPK/Akt signaling pathway, increased cellular proliferation and decreased cell adhesion, as evidenced by a decrease in E-cadherin expression in breast cancer cells [67]. Therefore, blockade of the GRP78/Cripto-1 interaction might interfere with Cripto-1 oncogenic activity *in vitro* and might be a novel target for therapy in tumors overexpressing Cripto-1. As discussed by the authors in the patent US0135904, the interaction between Cripto-1 and GRP78 may be selectively inhibited by antibodies that can either interact with Cripto-1 or with GRP78 [64]. The anti-GRP78 N-20 goat polyclonal antibody, commercially available from Santa Cruz Biotechnology, binds to a N-terminal region of GRP78 (aa 19–68) that has been demonstrated to directly interact with Cripto-1. Therefore, N-20 and Cripto-1 share the same binding site on GRP78 and they compete for GRP78 binding. The N-20 antibody is indeed effective in inhibiting Cripto-1 dependent Akt/MAPK phosphorylation and cellular proliferation of NCCIT embryonal carcinoma cells [67]. Anti-Cripto-1 antibodies targeting the CFC-domain of Cripto-1, which interacts with GRP78, may also represent good candidates to disrupt GRP78/Cripto-1 interaction (Fig. 1). For potential application of these blocking antibodies in human subjects, the authors discuss the possibility of generation of humanized forms of Cripto-1 or GRP78 blocking antibodies and the possibility to combine blocking antibodies targeting GRP78/Cripto-1 interaction with chemotherapeutic agents. Alternatively, they suggest that labeling of GRP78 or Cripto-1 targeting antibodies with radioactive isotopes or fluoro-chromes might be useful for diagnostic applications. In addition, a peptide or protein that could interfere with the interaction between Cripto-1 and GRP78 might also be used to inhibit the oncogenic signaling of the Cripto-1/GRP78 complex. This peptide might be identified by screening a phage display library of peptides in cells *in vitro* or a specific peptide corresponding to the

N-20 epitope of GRP78 might be used to bind Cripto-1 thereby preventing its interaction with GRP78.

6.3 Cancer vaccines

Cripto-1 is a cell surface protein unique to cancer cells that might be used as a target for immunotherapy. Two different immunotherapeutic approaches have been proposed to target Cripto-1 in different types of human tumors [68]. In the patent WO2008040759 by Pharmexa, the authors propose to generate an antibody based immunoresponse to Cripto-1 overexpressing cancer cells following a preventive or therapeutic immunization [69]. To generate an immunoresponse against the self-protein Cripto-1, they generated Cripto-1 molecules which contained a foreign peptide (PADRE) that can be recognized by the T helper lymphocytes. The Cripto-1 molecules containing PADRE (Cripto-1 Autovac) could be used for a preventive or therapeutic immunization by stimulating B cells to produce anti-Cripto-1 antibodies that could target and block Cripto-1 functions in cancer cells. In contrast, GlaxoSmithKline, in the patent WO16413A2, has proposed a different approach to target Cripto-1 using a vaccine based method in cancer patients with tumors overexpressing Cripto-1 [70]. The authors, using Cripto-1 immunogenic polypeptides, propose to generate a specific T cell response by incubating CD4⁺ and/or CD8⁺ T cells isolated from patients with tumors that are positive for Cripto-1 expression. Proliferating T cells stimulated by the Cripto-1 immunogenic peptides could be then reintroduced into patients to target specifically Cripto-1 expressing tumor cells. Finally, the peptide corresponding to a portion of the EGF-like domain of Cripto-1 protein that has been successfully used by Xing and colleagues, at the Austin Research Institute in Australia, to generate anti Cripto-1 rat monoclonal antibodies described earlier in this review, has been used to immunize BALB/c mice [49]. The immunized mice were protected from a challenge of syngeneic murine mammary tumor cell lines. Interestingly, while high levels of antibodies directed against Cripto-1 were detected in the immunized mice, a cytotoxic T cell response in these mice could not be detected.

7. Targeting Cripto-1 beyond cancer

7.1 Neurodegenerative diseases

Previous studies in embryonic stem (ES) cells have demonstrated that Cripto-1 is necessary for directing the differentiation of ES cells into cardiomyocytes while Cripto-1 negatively regulates neural differentiation [71]. Indeed, disruption of Cripto-1 expression in mouse ES cells enhances neurogenesis and midbrain dopaminergic differentiation [72,73]. These results suggest that blocking Cripto-1 expression or activity may represent a novel tool for cell replacement therapy in neurodegenerative diseases, such as Parkinson disease. The symptoms that characterize Parkinson disease are caused by the progressive degeneration of the dopaminergic neurons of the substantia nigra. Therefore methods that channel undifferentiated ES cells into dopaminergic neurons are under extensive evaluation, aiming to improve the safety of the ES cell grafting that often results in uncontrolled proliferation and teratoma formation [74,75]. Gabriella Minchiotti's laboratory, at the Institute of Genetics and Biophysics in Naples, Italy, using a rat animal model of Parkinson disease has demonstrated that mouse ES cells that are null for Cripto-1 expression (Cr-1^{-/-} ES cells), when grafted at low density into rats differentiate into neuronal cells in the brain and are able to restore the normal behavior in Parkinsonian rats without producing teratomas [72]. Recently, Minchiotti's laboratory, after screening a random combinatorial tetrameric tripeptide library synthesized using non natural peptides, has identified a tetrameric tripeptide (Cripto-1 Blocking Peptide/CBP) that antagonizes interaction of Cripto-1 with Alk4 (Fig. 1) [76]. The Cripto-1 Blocking Peptide was able to enhance dopaminergic differentiation of mouse ES cells *in vitro*. Furthermore, engraftment of ES cells treated with

Cripto-1 Blocking Peptide into the rat animal model of Parkinson disease resulted in functional recovery and behavioral improvement of Parkinsonian rats. Teratoma formation was, however, not completely abolished in rats receiving ES cells pretreated with the Cripto-1 Blocking Peptide, but was significantly reduced by approximately 60% as compared to rats implanted with ES cells treated with a control peptide. Further implications of Cripto-1 in neurodegenerative diseases derive the observations of Nancy Berman's group at the Kansas Medical Center. In this regard, Cripto-1 was found to be expressed at high levels in the brain of Macaques monkey that had been infected with a chimeric simian human immunodeficiency virus (SHIV) [77]. SHIV enters the central nervous system early after inoculation and causes encephalitis, characterized by transient meningitis and astrogliosis. Immunohistochemical analysis and RT-PCR confirmed widespread expression of Cripto-1 in the neurons of the central nervous system. Although the role of Cripto-1 during the neurodegenerative disease associated with SHIV infection is unclear, the authors, in the patent US2007122813, propose to target Cripto-1 with various approaches to inhibit progression of neurodegenerative diseases in mammals [78].

7.2 Degenerative muscle diseases

Degenerative muscle diseases, such as muscular dystrophy and spinal muscular atrophy, are characterized by skeletal muscle loss and few therapeutic approaches are available to restore the function of the lost muscle tissue [79]. In the patent WO 2008028888 by Peter Carmeliet and Gabriella Minichiotti, the authors claim that Cripto-1 might be used to treat muscle degenerative diseases [80]. Cripto-1 was in fact detected in myoblast cells of regenerative muscles and in primary myogenic satellite precursor cells in culture, whereas no expression of Cripto-1 was found in normal muscle fibers. A Cripto-1 recombinant protein induced a dose-dependent increase in proliferation and migration of myogenic precursor cells *in vitro*. In an *in vivo* experiment, a replication deficient adenovirus was used to overexpress a secreted form of Cripto-1 in skeletal muscles after hind limb ischemia or snake venom injection in gastrocnemius and tibial anterior muscles. Higher levels of regeneration and smaller damaged areas were observed in muscles overexpressing Cripto-1 as compared to control adenovirus treated animals. In addition, Cripto-1 overexpression also enhanced muscle vascularization and reduced fibrosis [20].

Expert opinion—Cripto-1 is a typical example of a gene that is associated with early embryogenesis and is also frequently expressed in a variety of human tumors in an aberrant spatial and temporal manner [1]. Emerging evidence has clearly shown the expression of Cripto-1 and other markers in different tumor types within a subset of cells with stem-like cell characteristics, also known as cancer stem cells or tumor initiating cells [81–86]. Cancer stem cells are capable of self-renewal and regeneration of the original phenotype of the tumor when implanted into immunodeficient mice. Cancer stem cells are generally resistant to chemo- and radio-therapy. Therefore, the survival capacity of tumor initiating cells together with their innate resistance to conventional therapy represents a major obstacle in the complete eradication of tumors. An ideal therapeutic approach in cancer patients would therefore aim to eliminate this undifferentiated population of tumor cells with stem-like characteristics, in order to completely eradicate the tumor mass. Recent evidence from our lab has shown that Cripto-1 is preferentially expressed in a subpopulation of human embryonal carcinoma cells with stem-like characteristics and is modulated by key regulators of pluripotent stem cells, such as Oct4 and Nanog [84]. A similar situation has been described in human melanomas and prostate cancer cells, where Cripto-1 is expressed in a subset of cancer stem-like cells [85,86]. Therefore, the efforts in the last several years to target Cripto-1 in cancer using different approaches have the potential to make a significant breakthrough in cancer research. Very promising are the humanized anti-Cripto-1 antibodies developed by Biogen-Idec that have now reached the clinic with a Phase I clinical trial.

Biogen-Idec's approach in choosing an anti Cripto-1 non-blocking antibody might be successful, allowing the high specificity of the anti-Cripto-1 antibody to deliver a potent cytotoxin to all the tumor cells expressing Cripto-1, including also the chemo- and radio-resistant subpopulation of tumor initiating cells. Although *in vitro* and *in vivo* data using rat anti-Cripto-1 monoclonal antibodies developed by the research group at the Austin Research Institute in Australia are impressive, future development in an agent that might be utilized in a clinical context might be challenging [61]. In fact, these rat anti-Cripto-1 monoclonal antibodies are IgM and not IgG, like the monoclonal anti-Cripto-1 antibodies developed by Biogen-Idec. Although the authors claim that the structure of the IgM in the rat anti-Cripto-1 monoclonal might contribute to the high efficiency of these antibodies in reducing tumor growth *in vitro* and *in vivo*, IgG monoclonal antibodies targeting Cripto-1 are preferable for future humanization and testing in cancer patients. Interestingly, they also demonstrate that Cripto-1 is highly expressed in a multidrug resistant leukemia cell line that overexpress the P-glycoprotein encoded by MDR1 gene [63]. This evidence further supports our hypothesis that Cripto-1 is expressed in a subpopulation of tumor cells that are highly resistant to conventional chemotherapy, but are sensitive to anti-Cripto-1 blocking antibodies. Finally, as suggested by Peter Gray and colleagues, monoclonal antibodies that target the GRP78/Cripto-1 complex might also become effective therapeutic agents. Anti-CFC monoclonal antibodies developed by Biogen-Idec were more effective in reducing xenograft tumor growth *in vivo* as compared to the anti-EGF monoclonal antibodies [19]. Since the CFC domain of Cripto-1 interacts with GRP78, it is possible that the higher efficacy of anti-CFC monoclonal antibodies might be due in part to blockade of the GRP78/Cripto-1 interaction, in addition to interference with Alk4 and Activin growth inhibitory signaling [20]. In addition, Cripto-1 inhibitors (i.e AS anti-Cripto-1 oligonucleotides and rat anti-Cripto-1 blocking antibodies) have been shown to enhance the antitumor effect of a number of chemotherapeutic agents when used in combination therapies [51–56]. This might provide another way for utilizing Cripto-1 inhibitors in cancer treatment, enhancing the usefulness of Cripto-1 inhibitors in cancer therapy. Cripto-1 also represents an effective target for cancer vaccine and two different approaches to induce a humoral or T-cell immunoresponse against the tumor marker Cripto-1 are discussed in the recent patent literature [68]. However, the impact of a T-cell mediated response or a humoral response on tumors overexpressing Cripto-1 in cancer patients needs further investigation. In addition to oncology, Cripto-1 has become an attractive target for developing potential therapeutic agents in neurodegenerative conditions and in degenerative muscle diseases. The Cripto-1 Blocking Peptide developed by Gabriella Minchiotti's laboratory might have a significant impact on the treatment of neurodegenerative diseases, including Parkinson disease [76]. In conclusion, Cripto-1 signaling continues to draw considerable interest as a promising therapeutic target for developing agents not only in cancer but also in neurodegenerative or muscle degenerative diseases.

Article Highlights

- Cripto-1 is a cell membrane-anchored protein that together with the TGF- β -related peptide Nodal regulates gastrulation during early embryonic development.
- Cripto-1 is expressed at low levels in adult tissues while Cripto-1 expression significantly increases during oncogenic transformation.
- High levels of Cripto-1 expression have been detected in a variety of human tumors, including breast and colon cancer.
- Different approaches have been used to target Cripto-1 in cancer cells, such as antisense oligonucleotides and monoclonal blocking antibodies.

- A humanized anti-Cripto-1 antibody conjugated with a cytotoxin is currently being evaluated in a phase I clinical trial in cancer patients.

Cripto-1 is a promising therapeutic target not only in cancer but also in neurodegenerative and degenerative muscle diseases.

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Figure 1. Monoclonal antibodies and peptides targeting the human Cripto-1 protein
Biogen-Idec has developed mouse monoclonal antibodies (mAbs) that bind to the NH2-terminal region, EGF-like domain or CFC motif of Cripto-1. The NH2 mAbs are binding antibodies, whereas the mAbs targeting the EGF-like or CFC domains are blocking antibodies. The binding B3.F6.1 antibody has been humanized and conjugated to the toxin DM4 for future use in cancer patients. The anti-CFC mAbs might potentially block interaction of Cripto-1 with GRP78. C3, C4 and C13 rat IgM monoclonal antibodies bind to the EGF-like domain of Cripto-1 and block tumor growth by induction of apoptosis and inhibition of Akt signaling pathway. The tetrameric tripeptide Cripto-1 Blocking peptide (CBP) blocks interaction of Cripto-1 with Alk4 and improves dopaminergic differentiation of ES cells *in vitro* and *in vivo* in a rat model of Parkinson's disease.