Published in final edited form as:

Semin Cancer Biol. 2014 December ; 0: 51–58. doi:10.1016/j.semcancer.2014.08.003.

The multifaceted role of the embryonic gene Cripto-1 in cancer, stem cells and epithelial-mesenchymal transition

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Abstract

Cripto-1 (CR-1)/Teratocarcinoma-derived growth factor1 (TDGF-1) is a cell surface glycosylphosphatidylinositol (GPI)-linked glycoprotein that can function either *in cis* (autocrine) or *in trans* (paracrine). The cell membrane *cis* form is found in lipid rafts and endosomes while the *trans* acting form lacking the GPI anchor is soluble. As a member of the epidermal growth factor (EGF)/Cripto-1-FRL-1-Cryptic (CFC) family, CR-1 functions as an obligatory co-receptor for the transforming growth factor- β (TGF- β) family members, Nodal and growth and differentiation factors 1 and 3 (GDF1/3) by activating Alk4/Alk7 signaling pathways that involve Smads 2, 3 and 4. In addition, CR-1 can activate non-Smad-dependent signaling elements such as PI3K, Akt and MAPK. Both of these pathways depend upon the 78 kDa glucose regulated protein (GRP78). Finally, CR-1 can facilitate signaling through the canonical Wnt/ β -catenin and Notch/Cbf-1 pathways by functioning as a chaperone protein for LRP5/6 and Notch, respectively. CR-1 is essential for early embryonic development and maintains embryonic stem cell pluripotentiality. CR-1 performs an essential role in the etiology and progression of several types of human tumors where it is expressed in a population of cancer stem cells (CSCs) and facilitates epithelial-mesenchymal transition (EMT). In this context, CR-1 can significantly enhance tumor cell migration, invasion and angiogenesis. Collectively, these facts suggest that CR-1 may be an attractive target in the diagnosis, prognosis and therapy of several types of human cancer.

Keywords

Cancer; Stem cells; Epithelial-mesenchymal transition; GRP78; Cripto-1

1. Introduction

Tumors may be considered as caricatures of the process of normal embryonic development whereby oncogeny recapitulates ontogeny in an inappropriate spatiotemporal context [1, 2]. Specifically, the subversion and corruption of embryonic signaling pathways such as Wnt β -catenin, Notch/Cbf-1, Hedgehog/Gli and Nodal/CR-1 may be instrumental as drivers in the initiation and/or progression of multiple types of cancer especially if these pathways are operative in CSCs (the cells thought to propagate the tumor) or transit amplifying (TA) progenitor populations [2, 3]. The loss of appropriate genetic or epigenetic regulatory mechanisms that might occur in normal adult somatic tissue stem cells (SCs), TA cells or the surrounding niche cell populations is a likely contributor to the alteration in expression and/or aberrant activation of these embryonic signaling pathways observed in tumors. The consequences of these alterations may then lead to a disruption in the cell-cell communication between different tissue compartments (epithelial and stromal) and a loss in normal tissue architecture as mediated by the processes of EMT and mesenchymal-epithelial transition (MET).

The normal tissue microenvironment also has a significant influence on the suppression, initiation, and progression of tumor cells. For example, the embryonic microenvironment or the adult stem cell niche can reprogram tumor cells to acquire a more normal cellular lineage restriction and to differentiate [4, 5]. Reciprocally, the tumor microenvironment that consists of myeloid suppressor cells, mesenchymal stem cells which are derived from the bone marrow or surrounding cancer-associated fibroblasts (CAFs) can directly or indirectly through secreted factors reprogram SCs and induced-pluripotent stem cells (iPSCs) to acquire properties of CSCs or tumor initiating cells (TICs) [6, 7]. Identification of these factors that are expressed in cancer cells or by the surrounding niche compartment may provide unique drug targets for cancer therapy.

In this review, we discuss the novel biological properties of the embryonic gene CR-1 and the molecular signaling pathways that are regulated by CR-1 which may contribute to its pro-tumorigenic role in various types of cancer. The expression of CR-1 in potential CSCs or TICs suggests that CR-1 coupled with its capacity to facilitate EMT could prove to be an efficacious therapeutic target for the clinical management of malignant disease.

2. Structure and mechanisms regulating expression of Cripto-1

Cripto-1/TDGF-1 is the original member of the epidermal growth factor (EGF)-Cripto-1-FRL-1-Cryptic (CFC) family of vertebrate signaling molecules. It was initially isolated from human (CR-1) NTERA-2 and mouse (Cr-1) F9 undifferentiated teratocarcinoma cells [8]. Structurally, Cripto-1 is a cell membrane-associated protein containing signal sequences 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) modification [8–13]. Removal of the GPI anchor by GPI-phospholipase D creates a soluble form of biologically active Cripto-1 [14] (Fig 1). Although several studies have shown the presence of both Cripto-1 forms in multiple cell

lines and *in vivo*, biological activities that are differentially regulated by the soluble versus cell-associated form are not yet clearly delineated [11, 15].

Mechanisms that directly regulate CR-1 expression during embryogenesis and tumorigenesis are incompletely defined. However, our group has previously shown that the promoter region of the CR-1 gene contains Smad-binding elements and T-cell factor/lymphoid enhancer factor (Tcf/Lef)-binding elements [16, 17]. In addition, during cardiac differentiation, hypoxia directly enhances CR-1 expression through the binding of the transcriptional factor HIF-1 α to hypoxia-responsive elements within the CR-1 promoter [18]. CR-1 has also been identified as a primary target gene of the Wnt/ β -catenin signaling pathway in colon carcinoma cells [19]. Furthermore, Behrens and colleagues demonstrated that the CR-1 promoter contains Nkx2-5 binding elements and that Nkx2-5 transcriptionally activates the CR-1 gene in early cardiac progenitors thereby regulating their maintenance and differentiation [20].

CR-1 is directly repressed by the orphan nuclear receptor germ cell nuclear factor (GCNF) during retinoic acid-induced differentiation of human embryonal carcinoma cells following binding of GCNF to a DR0 motif in the human CR-1 promoter region [21]. Bianco and colleagues found that LRH-1 orphan nuclear receptor binds to the CR-1 promoter and positively regulates CR-1 promoter luciferase activity in NTERA-2 cells and CR-1 gene expression in human embryonal and breast carcinoma cell lines as this relates to the methylation status of the CR-1 gene [22]. Interestingly, in *Xenopus* embryos, although xCR1 mRNA is equally distributed in all cells, xCR1 protein expression is restricted to the cells of the animal hemisphere [23]. This cell-specific translational repression mechanism is regulated through a specific element in the xCR1 mRNA 3'UTR called the TCE (translational control element) which binds Bicaudal-C RNA binding protein [24]. Recently, Chen and colleagues reported that in NSCLC (non-small cell lung cancer) tumors CR-1 is negatively regulated by the miR-15a/16 cluster [25]. Their results indicated that miR-15a-16 can repress CR-1 expression and luciferase activity through the wild-type CR-1 3'UTR which possesses a miR15a/16 binding element.

3. Role of Cripto-1 in embryogenesis and stem cell maintenance

During embryonic development in the mouse, Cr-1 is initially detected prior to gastrulation, in the inner cell mass and in extraembryonic trophoblast cells in the 4-day blastocyst. The highest Cr-1 expression is detected in epiblast cells undergoing EMT that are migrating and that give rise to the mesoderm and endoderm. Cr-1 and Cryptic signaling are 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 [26, 27]. Mouse embryos that lack the *Cr-1* gene (Cr-1 $^{-/-}$ mice) die at day 7.5 of embryogenesis due to defects in mesoderm formation and axial organization [27, 28]. After day 8 of embryogenesis, Cr-1 expression is restricted to the developing heart. Interestingly, genetic studies in humans have shown the involvement of CR-1 in the pathogenesis of ventricular septal defects, which is one of the most common congenital heart defects [29]. In adults, Cr-1 expression is significantly reduced and is probably sequestered to the stem cell compartment of adult tissues [30].

Cripto-1 is an established regulator of embryonic stem (ES) cells and iPSCs. Together with Nanog, Oct4 and connexin 43, Cripto-1 has been recognized as a potential stem cell marker [30]. Cripto-1 was found as a direct downstream target gene of Oct-4 and Nanog [31]. Reciprocally, Cripto-1, Nodal and Activin are essential in initiating and maintaining the expression of Nanog and Oct-4 [32]. Cripto-1 re-expression was detected with other ES cell genes during reprogramming in iPSCs that are derived from adult differentiated cells [33, 34]. Furthermore, Cripto-1 is involved in a molecular mechanism by which ES cells specify the neural lineage. Boles and colleagues identified a novel binding partner of Cr-1, the neuronal pentraxin 1 (NPTX-1), a secreted protein that is transiently released from differentiating ES cells and that is critical for neural induction [35]. NPTX-1 directly binds to Cripto-1 and inhibits both Nodal and BMP signaling. In the same context, Cr-1^{-/-} embryoid bodies spontaneously differentiate toward neurons, which opens new possibilities for cell replacement therapy in neurodegenerative diseases such as Parkinson's disease [36]. Cr-1 was also identified as a key factor in the hematopoietic stem cell niche. Together with the cofactor GRP78, Cr-1 has an essential role in the maintenance of dormant ES cells under hypoxia by regulating the HIF-1 complex [37] and in the maintenance of mammary stem cells *ex vivo* [38].

4. Cripto-1 interacting partners in cellular signaling

Cripto-1 has multiple binding partners and can modulate a variety of intracellular signaling pathways implicated in embryogenesis and oncogenic transformation (Fig 1). During embryogenesis, Cripto-1 functions primarily as a coreceptor for the TGF- β family ligands Nodal and growth and differentiation factors (GDFs) 1 and 3, leading to the activation of type I (Alk4/Alk7) serine-threonine kinase receptors and the Activin type II receptor complex, which triggers both the phosphorylation and activation of the Smad-2/Smad-3 intracellular signaling pathway. Cripto-1 also interacts with other TGF- β family members such as TGF- β 1 and Activins A and B, interfering with the binding of these ligands to their cognate receptors and attenuating TGF- β 1 and Activin A and B signaling in multiple cell lines. In addition to functioning as a co-receptor for Nodal, Cripto-1 can also function as a ligand for Glypican-1 which is a GPI-anchored heparan sulfate proteoglycan tethered to the plasma membrane within lipid raft microdomains. Binding of Cripto-1 to Glypican-1 activates the c-src/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (Akt) signaling pathways that regulate cell proliferation, motility, and survival [39].

There may be considerable cross-talk between Cripto-1 and the Wnt signaling pathway. For instance, phosphorylated Smad-2 can activate the Wnt/ β -catenin signaling pathway independently of Smad-4 through p300 [40]. It was recently discovered by Nagaoka et al., that in addition to being a transcriptional target of the canonical Wnt pathway, CR-1 modulates the canonical Wnt/ β -catenin/Tcf pathway by binding to the Wnt co-receptors low-density lipoprotein receptor-related protein (LRP) 5 and LRP6, which then facilitates their binding to Wnt3a [41]. CR-1 thereby functionally enhances Wnt3a signaling through cytoplasmic stabilization of β -catenin and elevated β -catenin/Tcf transcriptional activation. Another pathway affected by CR-1 is the canonical Notch signaling pathway. The Notch-1/Cbf-1 pathway is required for Nodal expression around the node and for proper left-right

determination in mouse and zebrafish [42, 43]. Notch4 can also regulate the expression of Nodal in malignant melanoma cells, indicating a cross-talk between the Nodal/Cripto-1 and Notch pathways during tumorigenesis [44]. Furthermore, CR-1 is also involved in the regulation of Notch receptor processing by enhancing the cleavage of the Notch1 precursor in the extracellular domain through a furin-1 protease in the ER/Golgi complex thereby potentiating Notch signaling [45].

Another molecule that might play a role in Cripto-1 signaling is exemplified by Tomoregulin-1 [46]. Tomoregulin-1 can specifically bind with low affinity to the erbB4 receptor [47]. Since Cripto-1 can indirectly enhance the tyrosine phosphorylation of erbB4 in breast cancer cells, interaction between Cripto-1 and Tomoregulin-1 might be important for phosphorylation and activation of erbB4 in mammalian cells [48]. Furthermore, CR-1 has been shown to interact with Caveolin-1 (Cav-1) in a specialized type of lipid raft, caveolae. Cav-1 acts as an inhibitor of Cripto-1 function, interfering with Cripto-1 activation of c-src and MAPK signaling pathways and reducing migration, invasion, and formation of branching structures stimulated by Cripto-1 in mouse mammary epithelial cells [49]. Lefty is another known Cripto-1 binding partner that functions as a co-receptor antagonist by binding to Cripto-1 and preventing Nodal from binding to its receptor complex. Lefty, like Cripto-1 is highly enriched in stem cells [50, 51]. Interestingly, Lefty that is secreted from human ES cells can down regulate Nodal signaling in metastatic melanoma and breast carcinoma cells thereby reducing their tumorigenic phenotype [44, 49]. Cripto-1 can also regulate another pathway as was shown by D'Aniello and colleagues. They demonstrated that Cr-1 null ES cells, which are unable to differentiate into a cardiac lineage, can be rescued by Apelin [52]. Apelin is a peptide identified as the ligand of the orphan G protein-coupled receptor related to Angiotensin-type I receptor (APJ) and regulates body fluid homeostasis, cardiovascular function and angiogenesis. CR-1 was found to directly stimulate the expression of Apelin and APJ. Furthermore, the Nodal/Cripto-1/Smad-4 pathway is also involved in promoting the expression of the hypertrophic response gene atrial natriuretic peptide (ANP) by activating the expression of the PITX2C transcription factor thereby contributing to myocardial hypertrophy [53].

A protein-protein interaction screen using Cripto-1 as bait led to the identification of Glucose Regulated Protein 78 kDa (GRP78) as a cell surface Cripto-1 binding partner [54, 55] and signaling co-factor [54]. GRP78 is an HSP70 family member that regulates protein folding and the unfolded protein response in the ER and its levels are highly elevated in response to nutrient deprivation, hypoxia and ER stress [56]. A fraction of GRP78 can localize to the cell surface where it mediates signaling by several growth stimulatory and cytostatic/cytotoxic effectors [57, 58]. Cell surface levels of GRP78 are high in stem cells and tumor cells and the elevated GRP78 levels in human tumors are closely associated with promotion of tumor growth, malignancy and therapy resistance [56, 59, 60]. Cripto-1 binding to GRP78 may be generally required for Cripto-1 function since treatment with a GRP78-directed antibody (N-20) that competitively blocks Cripto-1/GRP78 binding, or GRP78 knockdown, blocks Cripto-1 effects on Activin A, Activin B, TGF- β 1 and Nodal signaling and also inhibits soluble Cripto-1-dependent activation of c-src, Erk/MAPK and PI3K/Akt pathways [61]. More recent studies indicate that soluble Cripto-1 signals via cell surface GRP78 to promote maintenance of hematopoietic stem cells [37] and both fetal and

adult mammary stem cells [38]. In both of these studies, soluble Cripto-1 was shown to selectively regulate cells expressing high levels of surface GRP78, to activate the PI3K/Akt pathway and to promote stem cell maintenance *ex vivo*. Furthermore, treatment of cells with the neutralizing GRP78 N-20 antibody or ALK4^{L75A}-Fc, which acts as a soluble Cripto-1 “ligand trap” caused loss of the stem cell phenotype [37, 38]. These studies raise the possibility that Cripto-1 and GRP78 coordinately regulate stem cell function in other tissues and developmental contexts. Furthermore, since Cripto-1 and GRP78 are both highly expressed in a wide variety of human tumors, their cooperative promotion of stem cell properties in those settings, particularly in response to stresses that induce their expression, may promote tumor aggressiveness, plasticity and therapy resistance. In this regard, agents that disrupt Cripto-1/GRP78 signaling such as GRP78 neutralizing antibodies and ALK4^{L75A}-Fc [38] provide attractive therapeutic strategies.

5. Cripto-1: EMT and cancer stem cells

Broadly defined, CSCs are subsets of cells in various tumors that exhibit enhanced tumorigenicity in experimental settings and which are able to reestablish the cellular heterogeneity of the original tumor [62, 63]. CSCs, also known as tumor initiating cells, share several characteristics that have been associated with normal tissue SCs [64]. CSCs were first described in tumors of hematopoietic origin [62, 63] and have now been identified in several types of solid tumors, including cancers arising in the breast [65], lung [66], prostate [67], colon [68, 69], brain [70], head and neck [71], pancreas [72] and skin [73]. Long-term self-renewal potential, quiescence and resistance to chemotherapy and radiotherapy are properties associated with CSCs [74]. The activation of an EMT program is a fundamental step for morphogenesis during embryonic development that has parallels during tumor progression and metastasis that has also been associated with CSCs [75]. EMT is marked by an alteration of tissue organization with the loss of epithelial properties and the acquisition of a mesenchymal phenotype allowing for a gain in cell motility which enables mesenchymal cells to invade adjacent tissues. The reverse process, termed MET, is believed to participate in the establishment of distant metastases by allowing cancer cells to regain epithelial features and to colonize and integrate into distant organs [76]. While it remains unclear whether cancer stem cells are strictly dependent on regulatory pathways governing normal tissue stem cells, a number of pathways and mechanisms common to normal stem cells, early embryonic development, organogenesis, wound healing, and various stages of tumor progression have been elucidated [77–80]. The coordinated actions of EMT, other types of cellular developmental plasticity and other properties of stemness might even lead to a more aggressive tumor phenotype [81–84].

CR-1 is an example of a gene that has been shown to play a role in normal stem cells and during EMT, and has also been found to be expressed in a CSC subpopulation contributing to early cancer progression [85, 86]. In the embryo, Cr-1 is detected at high levels during gastrulation, when epiblastic cells undergo EMT, facilitating their migration through the primitive streak and eventually giving rise to the mesoderm and endoderm [30]. CR-1 has also been shown to promote EMT, migration, invasion and branching morphogenesis *in vitro* in mouse mammary epithelial cells and *in vivo* in mammary gland hyperplasias and in tumors derived from MMTV-CR-1 transgenic mice [87–89]. Moreover, NMuMG mouse

mammary epithelial cells that overexpress the transcription factor Msx2 undergo morphological and molecular modifications that are commonly associated with EMT. Interestingly, an increase in Cr-1 expression was detected in NMuMG Msx2-transfected cells suggesting that Cr-1 might promote EMT in these cells [90]. Furthermore, CR-1 is involved in tumor epithelial cell plasticity and may be an important EMT regulator in conjunction with Snail, Slug, Twist, and Six1 [91]. In this context, CR-1 can significantly enhance Snail expression in mammary epithelial cells [87]. Noteworthy, CR-1 is enriched in a subpopulation of cancer cells with stem-like characteristics. Recent evidence has demonstrated the presence of two distinct subpopulations of cells possessing high and low levels of CR-1 expression in human embryonal carcinoma (EC) cells [92], pluripotent stem cells derived from germ cell teratocarcinomas. Interestingly, both subpopulations behaved differently showing distinct gene expression profiles and differences *in vitro* and *in vivo* with respect to oncogenic competency. The EC cell fraction containing high levels of CR-1 formed tumor spheres in a serum-free suspension culture with an efficiency significantly higher than the CR-1 low-expressing EC cells. In addition, when injected subcutaneously into nude mice, the CR-1 high-expressing EC cells were able to generate tumors that were larger in size and with a shorter tumor latency period compared with tumors derived from CR-1 low-expressing cells [92]. In the same context, components of the Nodal/CR-1 signaling pathway were found to be overexpressed in pancreatic stem cells which regulated self-renewal and *in vivo* tumorigenicity [93]. Blocking the Alk4/7 receptor reversed the chemoresistance of the pancreatic CSCs. Moreover, CR-1 has also been identified in a CSC population of hormone-responsive and refractory human prostate tumor cell lines having distinct patterns of androgen metabolism, supporting a potential role for this population in prostate oncogenesis and tumor progression [94]. Additionally, a small subpopulation of CR-1 expressing cells was isolated from metastatic melanoma cells and was identified as a marker for CSCs in melanoma [86]. Finally, a recent report described three signaling pathways namely canonical Wnt, non-canonical Wnt and TGF- β , which induce an EMT program and subsequently function in an autocrine manner to maintain the mesenchymal stem cell state [95]. Remarkably, CR-1, together with other TGF- β and Wnt family members and proangiogenic factors, was among the reported secreted proteins present in the culture medium of immortalized human mammary epithelial cells that had undergone EMT and expressed phenotypic properties of CSCs.

6. Cripto-1 in transformation, migration, invasion and angiogenesis

Reactivation of certain signaling pathways that are crucial during embryonic development might induce cellular transformation and tumor progression in adult tissues [96]. CR-1 is a typical example of an embryonic gene that is re-expressed during tumorigenesis, functioning as an oncogene and driving cellular proliferation, migration, and invasion, as well as stimulating tumor angiogenesis *in vitro* and *in vivo* [30, 97]. CR-1 was first demonstrated to induce cellular transformation *in vitro* in mouse mammary epithelial cells and mouse embryonic fibroblasts, which acquired a transformed phenotype after being transfected with a CR-1 expression vector, as assessed by their ability to grow in an anchorage-independent manner in soft agar [85]. Furthermore, the involvement of Cripto-1 in tumor progression was shown by its ability to enhance migration and invasion of a variety of normal mammary

epithelial cells, MCF7 human breast cancer cells, and CaSki human cervical carcinoma cells. CR-1 was able to induce the expression of vimentin in CaSki cells suggesting that it may contribute to the invasive mesenchymal phenotype acquired by these cells. Interestingly, CR-1 expression was significantly increased in rat embryo fibroblasts or Fischer rat thyroid cells transformed by different oncogenes, such as c-Ha-ras or c-Ki-ras [85]. Furthermore, v-ras/Smad-7-transformed keratinocytes develop skin tumors that overexpress Cr-1 [98], suggesting that Smad-7-induced tumor formation may require upregulation of Cr-1 and other EGF-related peptides. Evidence also suggests that CR-1 might also modulate tumor angiogenesis, as demonstrated by Bianco and colleagues, where CR-1 was able to enhance the proliferation, migration and invasion of human umbilical endothelial cells, and stimulated their differentiation into vascular-like structures in Matrigel [99]. Similarly, overexpression of CR-1 in MCF-7 breast cancer cell xenografts enhanced tumor neovascularization *in vivo* [99]. It is possible that low oxygen levels trigger CR-1 expression within tumors, thereby inducing microvessel formation to sustain tumor growth. This in fact seems likely since, as alluded to above, it has been reported that hypoxic conditions can enhance CR-1 expression in human embryonal carcinoma cells that is mediated by the direct binding of HIF-1 α to the CR-1 promoter [18].

CR-1 can also function as an oncogene *in vivo* through possible cross-talk with other signaling pathways to promote mammary tumorigenesis. For example, there is a significant increase in Cr-1 expression in mammary tumors derived from transgenic mice overexpressing the oncogenes, neu (erbB-2), TGF- α , Int-3, polyoma middle T (PyMT) or simian virus 40 large T antigens [100]. A human CR-1 transgene has also been shown to directly promote mammary hyperplasias and adenocarcinomas of the mammary gland in transgenic mouse models overexpressing the human CR-1 transgene in mouse mammary glands under the control of the mouse mammary tumor virus (MMTV) or the whey acidic protein (WAP) promoters [89, 101]. The majority of nulliparous MMTV-CR-1 transgenic mice exhibit enhanced ductal branching, intraductal hyperplasias and hyperplastic alveolar nodules, and at least 30% of multiparous females develop multifocal hyperplasias and papillary adenocarcinomas. The relatively long latency period of tumor formation implies that additional genetic alterations and/or cross-talk with other signaling pathways such as Wnt/ β -catenin are necessary to induce mammary tumor formation. In fact, Strizzi and colleagues reported that the expression of the active form of β -catenin, dephosphorylated (DP)- β -catenin, was significantly increased in multiparous MMTV-CR-1 mammary tumors as compared to mammary tissue from control FVB/N mice [87]. They also found increased expression of phosphorylated (P)-c-src, P-focal adhesion kinase (FAK), P-Akt, P-glycogen synthase kinase 3 β (GSK3 β), and integrins α 3, α v, β 1, β 3, and β 4 in MMTV-CR-1 tumors, suggesting that CR-1 might play an important role in facilitating proliferation, migration and invasion of tumor cells *in vivo*. High levels of N-cadherin, vimentin, cyclin-D1, Snail, α -smooth muscle actin and fibronectin, and low levels of E-cadherin were also found in these CR-1 overexpressing tumors [87]. In addition to mammary tumors, 20% of MMTV-CR-1 females also developed uterine leiomyosarcomas after two years, and high levels of (P)-c-src, P-Akt, P-GSK3 β and DP- β -catenin as well as nuclear β -catenin were found in these uterine tumors, when compared to uteri from control mice [102]. This evidence suggests that CR-1 can facilitate mammary and uterine tumorigenesis by either activating c-src/Akt and/or

via cross-talk with the canonical Wnt/ β -catenin signaling pathway. Similarly, almost 50% of aged nulliparous WAP-CR-1 mice develop multifocal intraductal hyperplasias, and more than a half of multiparous WAP-CR-1 females develop mammary tumors of mixed histological subtypes, representing glandular, papillary and undifferentiated carcinoma, myoepithelioma and adeno-squamous carcinomas [101]. Like the MMTV-CR-1 mice, hyperactivation of the canonical Wnt/ β -catenin pathway was detected in WAP-CR-1 mammary tumors. As mentioned previously, activation of the Wnt/ β -catenin pathway during early mouse embryogenesis and in human colon carcinoma cells can enhance CR-1 expression [16, 19].

7. Expression of Cripto-1 in human carcinomas and premalignant lesions

As previously discussed in this review, CR-1 is not significantly expressed at significant levels in adult somatic tissues, with the possible exception of the tissue SC compartment, and its re-expression can be observed during oncogenic transformation. In addition to functioning as an oncogene *in vitro* and *in vivo*, CR-1 overexpression is detected at the mRNA and protein levels in a wide variety of solid human tumors of non-neuronal origin, including those of the reproductive and gastrointestinal systems, and also lung, skin, nasopharynx and embryonal carcinomas [85]. Furthermore, soluble CR-1 levels are elevated in the plasma obtained from colon and breast carcinoma patients [103].

However, two studies have also recently detected CR-1 expression in brain cancer. In a study by Tysnes and colleagues, invasive and angiogenic xenograft samples obtained from patients with glioblastoma (GBM), showed elevated expression of CR-1 [104]. Additionally, patient samples from primary GBM showed significantly high levels of CR-1 protein as detected by immunohistochemistry, and the higher CR-1 scores were also associated with shorter survival in a subset of younger patients. In a more recent study, high CR-1 protein levels were detected in plasma from GBM cancer patients, which was significantly correlated with a shorter overall survival. Interestingly, CR-1 was detected in the perivascular areas of GBM cells, and also in endothelial cells [105]. For an extensive review on Cripto-1 expression in solid human tumors of non-neuronal origin, see [85, 97].

8. Cripto-1 as a therapeutic target in human cancer

Due to its intimate involvement in processes such as oncogenesis and EMT and minimal expression in adult tissues, CR-1 may be considered as an attractive target for therapeutic intervention. In particular, the association of CR-1 with CSCs is intriguing as this population of cells is intrinsically resistant to standard chemotherapy and radiotherapy [106]. Current strategies that can effectively target and neutralize the potential oncogenic effects of CR-1 include the use of antisense (AS) oligonucleotides, monoclonal antibodies (mAbs), inhibitory peptides, small molecule antagonists and antibodies directed against CR-1 binding partners (Fig 1). Antisense oligonucleotides targeting the expression of CR-1 have been effective in the inhibition of breast, colon and ovarian cancer cells *in vitro* alone and with greater effect when combined with AS oligonucleotides targeting other oncogenic growth factors, such as TGF- α and amphiregulin [107–110]. These same AS oligonucleotides have also shown efficacy *in vivo* by inhibiting the proliferation of colon cancer xenografts in nude

mice alone and with higher efficacy when combined with conventional chemotherapy or radiotherapy [108].

A number of mAbs directed against CR-1 have been used in various model systems with significant effects on reducing cellular proliferation and inhibiting tumor growth. Biogen-Idec has produced several mouse mAbs that target various regions within CR-1. A mAb (A8.G3.5) generated against the CFC motif has shown efficacy by inhibiting cancer cell proliferation *in vitro* and tumor xenografts *in vivo* by relieving the CR-1-mediated blockade of Activin B signaling and growth inhibition [111]. Antibodies targeting the EGF-like motif have also proven to be effective in inhibiting Nodal signaling by disrupting the interaction of CR-1 with Nodal [112]. Xing and colleagues have produced rat monoclonal IgM antibodies (C4, C13) that also target the EGF-like motif of CR-1 and that have proven to be highly effective in inhibiting the growth of breast, colon, lung, prostate and leukemic cancer cell lines *in vitro* [113]. In addition, these antibodies can inhibit the xenograft tumor growth of colon cancer cells *in vivo*, enhance the cytotoxic effects of chemotherapeutic agents, and can induce apoptosis in these systems and in multi-drug resistant leukemia cells [113, 114]. An Fc chimera consisting of the Alk4 extracellular domain fused to the Fc domain of human IgG also has antitumor potential since it blocked CR-1-induced proliferation, migration and stem cell maintenance [38]. Antibodies produced against the N-terminus of CR-1 (B3.F6.1 and A10.B2.18) have shown strong binding without neutralization of the biological activity of CR-1 [112, 115] and a humanized version of B3.F6.1 conjugated to a cytotoxin (DM4) has been used in a recently concluded phase I clinical trial in relapsed/refractory solid tumors with no current plans to continue [112, 116]. CR-1 binding partners can also be targeted for therapeutic intervention, such as GRP78. Disruption of the CR-1/GRP78 complex with an anti-GRP78 antibody has been effective in abrogating Akt/MAPK signaling in NCCIT cells [61] and elucidating the role of CR-1 in the maintenance of hematopoietic stem cells [37].

Other approaches have been used to neutralize CR-1 binding to the Activin/TGF- β signaling complex. Alantolactone, a natural small molecule derived from several plants [117], has been shown to impair the CR-1-mediated blockade of Activin signaling by disrupting the association of CR-1 with the Activin receptor type IIA [118], mimicking the effects of mAbs targeting the CFC motif of CR-1. Recently, a non-natural tetrameric tripeptide that binds the CR-1 CFC motif was found to enhance differentiation of mouse ES cells *in vitro* and improve neurological function in an *in vivo* rat model of Parkinson's disease [119]. This peptide has the potential to re-activate the Activin signaling complex in an oncogenic setting in a similar fashion as seen with alantolactone and CFC-targeting antibodies. Whether alone or in concert with other therapeutic regimens, the abrogation of CR-1 expression and binding to Activin/TGF- β signaling complex has significant therapeutic potential.

9. Conclusion and perspectives

The abnormal spatial and temporal reexpression of embryonic signaling genes at different stages of tumor development in a variety of human cancers is now a well-recognized fact. In particular, the subversion of these key regulatory genes in CSCs or transit amplifying progenitor cells in human cancers may be extremely deleterious for restricting tumor

progression and for preventing the re-emergence of secondary cancer following the use of primary chemo- and/or radiotherapy. Therefore, the targeting of embryonic genes that drive the maintenance or self-renewal of CSCs/TICs becomes attractive therapeutically. Conventional cancer therapies generally attack more fully differentiated and/or rapidly cycling tumor cells without significantly impeding the relatively small and quiescent population of more undifferentiated CSCs. Therapies that deplete the bulk tumor population combined with novel therapies that disrupt singular or multiple embryonic signaling pathways in CSCs, the CSC niche or processes such as EMT that initiate the formation of CSCs seems to be warranted for successfully and permanently eradicating tumors. CR-1/TDGF-1 is an example of one such embryonic gene that is expressed at significant levels in a relatively high proportion of human cancers. CR-1 is functionally an important nexus point for several different embryonic signaling pathways such as Nodal, Notch and Wnt/ β -catenin that have been implicated in regulating the etiology and progression of human tumors. The identification of upstream genes that regulate CR-1 expression and activity as well as downstream targets that are in turn regulated by CR-1 will significantly improve our understanding of the biology of this complex regulatory gene and hopefully expose other potential novel therapeutic targets in cancer.

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Figure 1.

The multi-faceted Cripto-1-interacting hub. Cripto-1, in yellow, interacts with various components of multiple signaling pathways to drive processes including stem cell renewal, EMT, proliferation and oncogenesis (green). Inhibitors and cellular components that counteract the oncogenic properties of Cripto-1 are shown in red. Above right, the domains of Cripto-1 are shown with therapeutic interactions. Interactions that are hypothesized, but not yet experimentally shown, are indicated by a “?”. Numbers represent the amino acid numbers at the beginning and end of each domain/region.