19

Cripto-1: At the Crossroads of Embryonic Stem Cells and Cancer

Nadia Pereira Castro, Maria Cristina Rangel, Tadahiro Nagaoka, Hideaki Karasawa, David S. Salomon and Caterina Bianco
Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, National Institutes of Health, Bethesda, USA

1. Introduction

Human Cripto-1 is a member of the Epidermal Growth Factor-Cripto-FRL-1-cryptic (EGF-CFC) family of peptides (Bianco et al., 2010; de Castro et al., 2010). During early vertebrate development, Cripto-1 functions as a co-receptor for transforming growth factor β (TGF-β) ligands, such as Nodal and growth and differentiation factor-1 and -3 (GDF-1 and GDF-3), through an heteromeric complex composed of Activin type II and type I (ALK4) serine threonine kinase receptors in the plasma membrane. Genetic studies in zebrafish and mice have demonstrated that Cripto-1/Nodal signaling is essential for the formation of the primitive streak, patterning of the anterior/posterior (A/P) axis, specification of mesoderm and endoderm and establishment of left/right (L/R) asymmetry (Bianco et al., 2010; de Castro et al., 2010). In adult tissues, Cripto-1 is expressed at low levels in all stages of mammary gland development and its expression increases during pregnancy and lactation. Overexpression of Cripto-1 in mouse mammary epithelial cells leads to their transformation *in vitro* and can enhance migration, invasion, branching morphogenesis and epithelial to mesenchymal transition (EMT) (Bianco et al., 2010; de Castro et al., 2010). Furthermore, transgenic mouse studies have shown that overexpression of a human Cripto-1 transgene in the mouse mammary gland under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter results in mammary hyperplasias and papillary adenocarcinomas (Wechselberger et al., 2005). Cripto-1 is also expressed at high levels in approximately 50-80% of different types of human carcinomas, including breast, colon, pancreas, lung and ovary (Bianco et al., 2010; de Castro et al., 2010). Cripto-1 is therefore an example of an embryonic gene that plays an important role not only during embryogenesis, but is also implicated in malignant progression. Furthermore, Cripto-1 signaling might represent a common signaling pathway shared by embryonic stem cells and cancer cells. Recent studies have shown that Cripto-1 is an important component of critical core pathways that regulate stem cell self-renewal and differentiation (Hough et al., 2009). Chromatin immunoprecipitation (ChiP) analysis has revealed the presence of Oct-4 and Nanog binding sites in the Cripto-1 promoter region, suggesting that Cripto-1 is a direct transcriptional target of Oct-4 and Nanog transcription factors (Loh et al., 2006). More importantly, several signaling pathways that are critical for early embryonic development and regulate stem cell

proliferation and differentiation have been shown to cross-talk with Cripto-1 (Bianco et al., 2010). Examples of embryonic signaling pathways that interact with Cripto-1 are the Wnt-β/catenin, the Notch, hypoxia and TGF-β signaling pathways. Interestingly, Cripto-1 and other embryonic genes that regulate stem cell function are also overexpressed in human tumors, confirming this connection between stem cells and cancer. Recently, Cripto-1 has been shown to be highly expressed in a small subset of stem-like cells in human embryonal carcinoma cells, in human malignant melanomas and in androgen-responsive and refractory human prostate cancer cells (Cocciadiferro et al., 2009; Strizzi et al., 2008; Watanabe et al., 2010). Therefore, Cripto-1 signaling pathway may represent an attractive target for treatment in cancer, because Cripto-1 targeting will eliminate not only differentiated cancer cells but also might target an undifferentiated subpopulation of tumor cells that exhibit stem-like characteristics, thereby leading to eradication of the tumor. In this review we will discuss the dual role of Cripto-1 as embryonic gene in the regulation of stem cell self-renewal and differentiation and as oncogene with re-expression in human cancers.

2. The EGF-CFC family

Human Cripto-1 is a member of the EGF-CFC family of peptides identified only in vertebrates, and plays an important role in embryonic development and in tumor progression. The EGF-CFC family includes monkey Cripto-1, mouse Cripto-1 (Cr-1=cfc2), chicken Cripto-1, zebrafish one-eyed pinhead (*oep*), *Xenopus* XCR1/FRL-1, XCR2 and XCR3, mouse cryptic (Cfc1) and human Cryptic (CFC1) (Bianco et al., 2005b). Structurally, these proteins contain an NH2-terminal signal peptide, a modified EGF-like domain, a CFC cysteine-rich domain and a short hydrophobic COOH-terminus containing short sequences for glycosylphosphatidylinositol (GPI) cleavage and attachment (Figure 1).

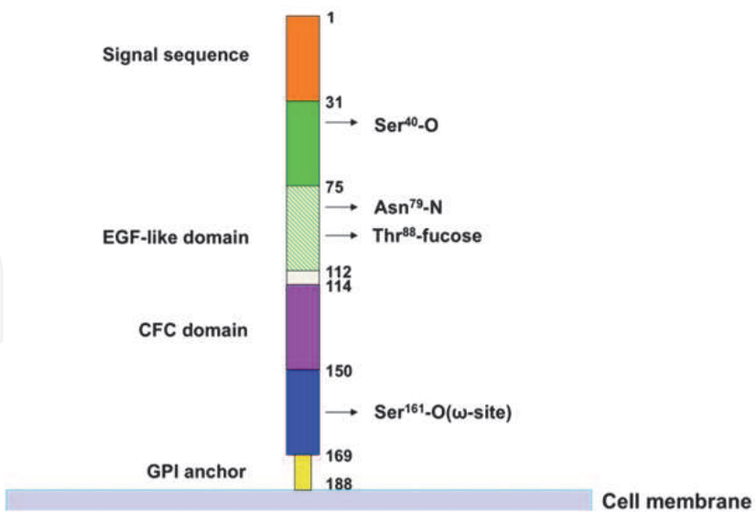

Fig. 1. Structure of the human Cripto-1 protein.

Cripto-1 contains several glycosylation sites and the residue Threonin 88 within the EGF-like domain modulates Cripto-1 ability to activate a Nodal-dependent signaling pathway. Cripto-1 can be cleaved from the cell membrane and can be released in the supernatant of cells by activity of the enzyme GPI-phospholipase D (GPI-PLD). EGF: epidermal growth

factor, CFC: Cripto-FRL-1-Cryptic, GPI: glycosylphosphatidylinositol, Ser: Serine, Asn: Asparagine, Thr: Threonine.

GPI anchoring determines membrane localization of Cripto-1 within lipid rafts microdomains and is required for the paracrine activity of Cripto-1 as a Nodal co-receptor (Bianco et al., 2008; Watanabe et al., 2007b). Removal of the GPI anchor by GPI-PLD generates a soluble form of Cripto-1, which can therefore function both as a cell membrane anchored protein or as a soluble protein (Watanabe et al., 2007a; Watanabe et al., 2007b). In fact, soluble forms of Cripto-1 are biologically active in a number of different *in vitro* and *in vivo* assays (Minchiotti et al., 2001; Xu et al., 1999; Yan et al., 2002). Furthermore, Cripto-1 protein has several glycosyl modification sites, including O-linked glycosylation at Ser40 and Ser161 (ω-site for GPI-attachment), N-linked glycosylation at Asn79 and O-linked fucosylation at Thr88 (Figure 1) (Minchiotti et al., 2000; Schiffer et al., 2001; Shi et al., 2007; Watanabe et al., 2007a; Watanabe et al., 2007b). Among them, O-linked fucosylation of EGF-CFC proteins is required for their ability to function as co-receptors for the TGF-β-related protein Nodal (Schiffer et al., 2001). However, Cripto-1 O-fucosylation mutants are fully functional with regard to activation of Nodal independent signaling pathways (Bianco et al., 2008). Another study has demonstrated that is the Thr88 residue and not fucosylation of this residue that is necessary for Cripto-1 to function as a Nodal co-receptor (Schiffer et al., 2001; Shi et al., 2007).

3. Cripto-1 during embryonic development

In the embryonic development EGF-CFC proteins function as co-receptors for the TGF-β ligands Nodal, GDF-1 and GDF-3 (Bianco et al., 2010; de Castro et al., 2010 as cited in Yeo et al. 2001; Andersson at al. 2007). Genetic studies in zebrafish and mice have defined an essential role for Nodal that functions through *oep*/Cripto-1 in the formation of the primitive streak, patterning of the A/P axis, and specification of mesoderm and endoderm (mesoendoderm) (Bianco et al., 2010; de Castro et al., 2010 as cited in Chu et al. 2005). In later stages of embryonic development the EGF-CFC ortholog of Cripto-1, Cryptic, regulates the establishment of L/R axis, allowing asymmetric development of visceral organs (Bianco et al., 2010 as cited in Yan et al., 1999). Biochemical studies have demonstrated that EGF-CFC proteins bind directly to Nodal, GDF-1 or GDF-3 and the Activin type I receptor ALK4 (ActRIB), recruiting the Activin type II receptor and inducing activation and phosphorylation of Smad-2/Smad-3 signaling pathway (Bianco et al., 2010) (Figure 2). However, Cripto-1 can also regulate A/P axis specification independently of Nodal signaling (D'Andrea et al., 2008). In fact, Cripto-1$^{F78A/F78A}$ mouse embryos carrying the amino acid substitution F78A are unable to activate a Nodal signaling pathway but clearly establish an A/P axis and initiate germ layer formation and gastrulation movements (D'Andrea et al., 2008). During early mouse embryogenesis, Cripto-1 cannot be detected until prior to the gastrulation stage in the inner cell mass and in extra-embryonic trophoblast cells at day 4 of development. Increase in Cripto-1 expression is observed on day 6.5 of embryonic development when Cripto-1 is found in the primitive streak within epiblast cells undergoing EMT, which eventually give rise to mesoderm and endoderm (Bianco et al., 2010; de Castro et al., 2010). Cripto-1 is also detected in the ectoplacental cone at this stage. Cripto-1 expression then decreases on day 7 when it is detected mostly in the truncus arterious of the developing heart.

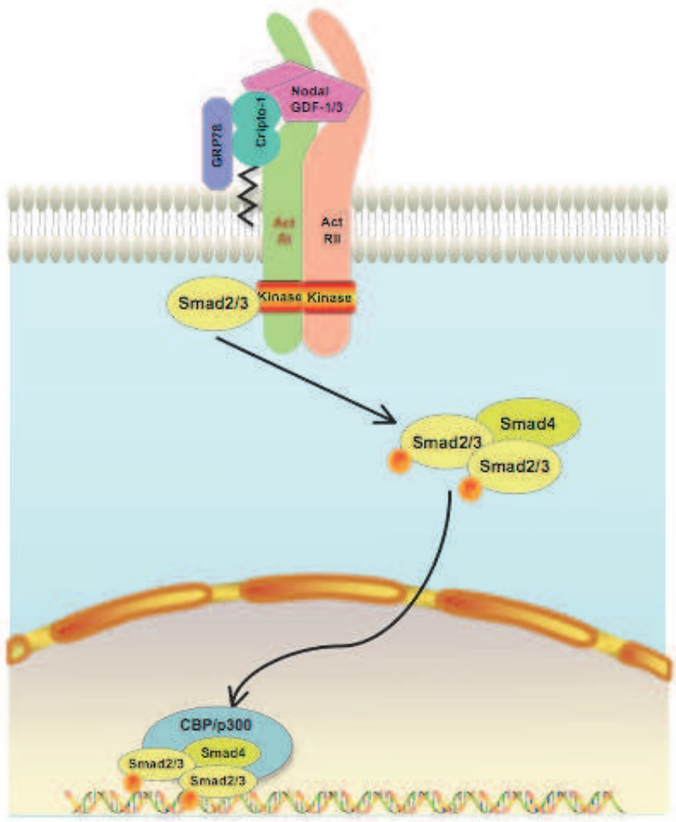

Fig. 2. Cripto-1/Nodal dependent signaling pathway.

Cripto-1 is a co-receptor for Nodal, GDF-1 and GDF-3, allowing them to interact with ALK4 (ActRIB). The Cripto-1/Nodal/ALK4/ActRII receptor complex triggers activation and phosphorylation of Smad-2 and Smad-3. Phosphorylated Smad-2 and Smad-3 form a complex with Smad-4 and they translocate into the nucleus. In the nucleus, Smad-2/-3/-4 complex interacts with CREB binding protein (CBP)/p300 and activates transcription of specific target genes. The heat shock protein GRP78 can also enhance Cripto-1/Nodal-dependent signaling pathway, as discussed later in the text. GDF-1/-3: growth and differentiation factor-1/3, GRP78: glucose-regulated protein 78.

With the exception of the developing heart, little if any expression of Cripto-1 can be detected in the embryo after day 8 (Bianco et al., 2010; de Castro et al., 2010 as cited in Dono et al., 1993; Minchiotti et al., 2000). Disruption of Cripto-1 in Cripto-1 $^{-/-}$ embryos is embryonically lethal and results in the formation of embryos that possess a head without a trunk, demonstrating that there is a severe deficiency in embryonic mesoderm and endoderm without a loss of anterior neuroectoderm formation (de Castro et al., 2010 as cited in Ding et al. 1998). Initiation of the primitive heart tube in Cripto-1 $^{-/-}$ mice is severely inhibited due to failure in the development of functional beating cardiomyocytes, as demonstrated by the absence of expression of terminal myocardial differentiation genes (de Castro et al., 2010 as cited in Xu et al., 1998). Cripto-1 $^{-/-}$ embryonic bodies (EB) derived from Cripto-1 $^{-/-}$ embryonic stem (ES) cells fail to form beating cardiomyocytes, while they differentiate into neuronal cells. Addition of a Cripto-1 recombinant protein to Cripto-1 $^{-/-}$

EBs during early time points (0–2 days) of differentiation is able to rescue cardiomyocyte differentiation. However, addition of Cripto-1 recombinant protein during later stages of differentiation fails to rescue cardiamyocyte differentiation, suggesting that Cripto-1 ability to promote cardiac differentiation of EB is restricted to an early window of this differentiation program (Minchiotti, 2005, as cited in Parisi et al., 2003). Interestingly, a microarray study revealed that Cripto-1 $^{-/-}$ ES cells had a reduced mRNA expression of the G protein coupled receptor APJ (also known as angiotensin type I-like receptor) and its ligand Apelin, as compared to control wild type ES cells. Gain of function experiments showed that APJ redirects the neural fate of Cripto-1 $^{-/-}$ ES cells and restores the cardiogenic program. Furthermore, comparison of Cripto-1, APJ and Apelin expression profile in mouse embryo by *in situ* hybridization revealed that expression of Apelin and APJ correlates with Cripto-1 expression. In fact, Apelin mRNA was clearly expressed in the developing primitive streak, whereas APJ was expressed in the primitive streak and adjacent mesoderm, resembling Cripto-1 expression pattern (D'Aniello et al., 2009). Therefore, APJ and Apelin are downstream targets of Cripto-1 signaling pathway and together with Cripto-1 they drive ES cells toward a cardiac lineage.

3.1 Cripto-1 in embryonic stem cells

Stem cells have the capacity to divide for an undetermined period of time and a potential to develop into many different cell types throughout early life and growth. Stem cells are distinguished from other cell types by two important characteristics. First, they possess the capability to differentiate into mature cells of any particular tissue (pluripotency). Second, they can undergo through numerous cell cycle divisions while maintaining their undifferentiated state (self-renewal). ES cells can be isolated from a 3- to 5-day-old embryo, called blastocyst, and have the potential to give rise to all specialized tissues and organs of a mature organism. Adult stem cells are found in various adult tissues, and function as a reservoir for cells that are lost during injury or disease (Bendall et al., 2008). Mouse embryonic stem cells (mES) or human embryonic stem cells (hES) have been very useful in the field of stem cell research. Comparison of gene expression profiles across species has shown that mouse and human ES cells share common highly conserved signaling pathways that regulate self-renewal and pluripotency, including the Cripto-1/Nodal signaling pathway. For example, in addition to Cripto-1, genes such as Oct-4, Lefty, Nodal, Sox-2, Utf-1 (undifferentiated embryonic cell transcription factor-1) and Tert (telomerase reverse transcriptase) are highly enriched in both mouse and human ES cells (Wei et al., 2005). Additionally, Cripto-1 has been identified as a pluripotency marker also in primate ES cells together with Oct-4, Nanog, Sox-2, Tert, LeftyA, and Rex-1 (Chang et al., 2010). In 2007 a comparative study of a large and diverse set of hES cell lines assessed the expression pattern of commonly used stem cell markers. All the hES cells analyzed exhibited similar expression profile for several stem cell markers, including the glycolipid stage specific embryonic antigens SSEA3 and SSEA4, the keratan sulfate antigens TRA-1-60, TRA-1-81, and the developmentally regulated genes including Nanog, Oct-4, Dnmt3b, Gabrb3, GDF-3 and Cripto-1 (Adewumi et al., 2007; Bianco et al., 2010; de Castro et al., 2010). Finally, Cripto-1 has been reported as a direct target gene of stem cell transcription factors (Bianco et al., 2010; de Castro et al., 2010; Hough et al., 2009). For instance, using the ChIP paired-end ditags method, Loh and collaborators mapped the binding sites of the transcription factors Oct-4 and Nanog in the mouse ES cell genome. Cripto-1 promoter was found to include Oct-4 and

Nanog binding sites, suggesting that key modulators of stem cell self-renewal and pluripotentiality directly regulate Cripto-1 expression in ES cells (Bianco et al., 2010; de Castro et al., 2010; Loh et al., 2006).

3.1.1 Cross-talk of Cripto-1 with stem cell signature pathways

Several signaling pathways regulate in a coordinate fashion early embryonic development and stem cell proliferation, maintenance and differentiation. Some of these signaling cascades have been shown to cross-talk with Cripto-1 signaling, suggesting a pivotal role played by Cripto-1 in stem cell self-renewal and maintenance. Among these signaling pathways are genes in the Wnt/β-catenin signaling pathway, TGF-β family members, the Notch pathway, and hypoxia inducible factor-1 alpha (HIF-1α) (Bianco et al., 2010; de Castro et al., 2010). A schematic diagram of the cross-talk of Cripto-1/Nodal signaling with other stem cell signature signaling pathways is shown in figure 3.

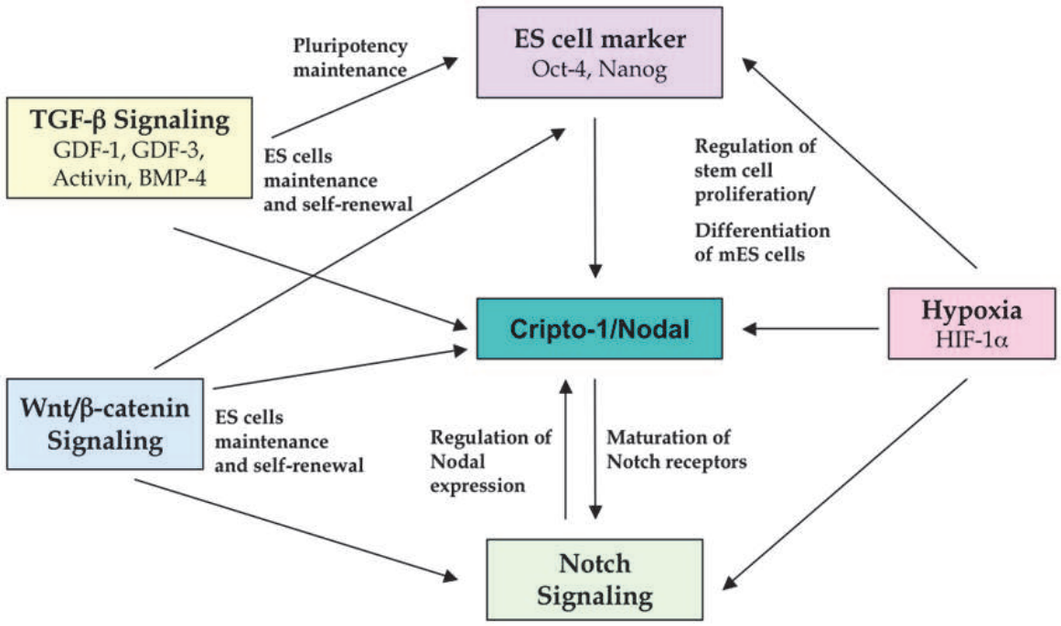

Fig. 3. Interplay of Cripto-1/Nodal signaling with other embryonic stem cell signaling pathways.

Cripto-1 signaling is a downstream target of Oct-4, Nanog, Wnt/β-catenin, Notch and Hypoxia/HIF-1α pathways. Cripto-1 also mediates signaling of TGF-β family members and enhances Notch signaling by facilitation of Notch receptor maturation. TGF-β: transforming growth factor-β, HIF-1α: hypoxia inducible factor-1α, GDF-1/-3: growth and differentiation factor-1/-3, BMP-4: bone morphogenetic protein-4, ES cells: embryonic stem cells, mES cells: mouse embryonic stem cells.

Activation of the canonical Wnt/β-catenin pathway has been reported to sustain the undifferentiated state of ES cells by triggering transcription of genes that regulate ES cell pluripotency and self-renewal, such as Oct-4, Nanog, Sox-2 and Cripto-1. For instance, Cripto-1 has been identified as a direct target gene in the canonical Wnt/β-catenin signaling pathway during embryonic development and in colon carcinoma cell lines and tissues

(Morkel et al., 2003). Remarkably, in β-catenin $^{-/-}$ or Wnt3 $^{-/-}$ mutant mouse embryos, Cripto-1 expression was found to be dramatically downregulated, suggesting that the Wnt/β-catenin signaling pathway is a positive regulator of Cripto-1 expression during embryonic development (Bianco et al., 2010). In addition, in *Xenopus*, the Cripto-1 ortholog XCR1/FRL-1 functions as a co-receptor for Wnt11 and together with frizzled receptor 7 and Glypican-4 mediates stabilization and activation of β-catenin (Bianco et al., 2010). Several studies have clearly implicated Wnt/β-catenin signaling pathway in initiation and maintenance of carcinomas of the skin, intestine, liver, and brain (Bianco et al., 2010; de Castro et al., 2010). Hence, the interaction between the Wnt/β-catenin and Cripto-1 signaling pathways might be significant also in cancer initiation and progression. Activation of TGF-β/Activin/Nodal signaling has also been reported to be required for the maintenance of undifferentiated state in human ES cells (James et al., 2005; Vallier et al., 2009). Some TGF-β family members, including Nodal, GDF-1 and GDF-3, require Cripto-1 for signaling and they are critical for ES cell maintenance and self-renewal (Bianco et al., 2010). Furthermore, Activin, TGF-β and bone morphogenetic protein (BMP)-4 can directly regulate Cripto-1 expression by binding to smad binding elements (SBE) within the Cripto-1 promoter in human embryonal carcinoma cells and in human colon cancer cells (Mancino et al., 2008). GDF-3 and Cripto-1 have also been identified as ES cell markers that are enriched in a population of ES cells which are uncommitted and have high self-renewal capacity (Hough et al., 2009). Finally, Activin/TGF-β signaling activity is required for Nanog expression in epiblast cells, suggesting a key role played by TGF-β family members in maintaining ES pluripotency and self-renewal (Shin et al., 2011; Wang et al., 2009). The Notch pathway is a key regulator of many developmental processes during fetal and adult differentiation. Studies have reported that inappropriate activation of Notch signaling cascade contributes to tumorigenesis (Wilson & Radtke, 2006; Wang et al., 2009). Interaction between Notch and Cripto-1/Nodal signaling pathways has also been described. Analysis of the transcriptional regulatory regions within the mouse Nodal promoter has identified the presence of CBF1 binding elements, suggesting a direct regulation of Nodal expression by Notch signaling (Bianco et al., 2010; as cited in Raya et al., 2003; Krebs et al 2003). In this regard, mouse embryo double mutants for Notch1 and Notch2 exhibit multiple defects in L/R asymmetry, which is regulated by Nodal. Moreover, a new insight into Notch and Cripto-1/Nodal signaling pathways has been reported. Watanabe and colleagues (2009) using a yeast two-hybrid system screened a mouse embryo or human colon cDNA prey library for potential Cripto-1 binding partners. Six candidate genes were isolated, including the mouse Notch3. By coimmunoprecipitation analysis Cripto-1 was found to directly bind to all four mammalian Notch receptors. Surprisingly, binding of Cripto-1 to Notch1 occurred mainly inside the cells in the endoplasmic reticulum/Golgi complex, where Cripto-1 enhanced cleavage of the Notch1 extracellular domain through a furin-like convertase. Enhanced cleavage of Notch1 receptor by Cripto-1 potentiates Notch signaling as demonstrated by enhanced ligand-induced activation of Notch signaling in Chinese hamster ovary cells expressing a CBF1-dependent Notch reporter assay (Watanabe et al., 2009). Hypoxia is an important regulator of stem cell proliferation and differentiation (Yeung et al., 2011). In fact, tissue stem cells reside within niches that are naturally hypoxic and low oxygen levels prevent their differentiation toward specific fates. Moreover, HIF-2α can directly regulate Oct-4 expression in ES cells by binding to hypoxia-responsive elements (HREs) within the promoter of mouse Oct-4. HIF-1α interacts with Notch signaling by binding to Notch

intracellular domain and enhancing transcription of Notch downstream target genes Hes-1 and Hey-2. HIF-1α, in addition to regulating Notch1 signaling, can also regulate Cripto-1 expression by binding to HREs within the promoter region of mouse and human Cripto-1 gene in mouse ES cells and in human embryonal carcinoma cells (Bianco et al., 2009). In addition, hypoxic conditions enhanced differentiation of mES cells into beating cardiomyocytes when compared with mES cells grown under normoxic conditions. However, expression of Cripto-1 in mES was critical, because hypoxia was unable to induce differentiation of mES cells into cardiomyocytes in the absence of Cripto-1 expression.

4. Cripto-1 in cancer

Similarities between embryonic development and cellular transformation during oncogenesis have led to the identification of common signaling pathways, suggesting that reactivation of developmental signaling pathways might drive cell transformation and tumor progression in adult tissues (Bianco et al., 2010). Cripto-1 is a typical example of an embryonic gene that is re-expressed in human tumors, promoting cellular proliferation, migration, and tumor angiogenesis (Figure 4).

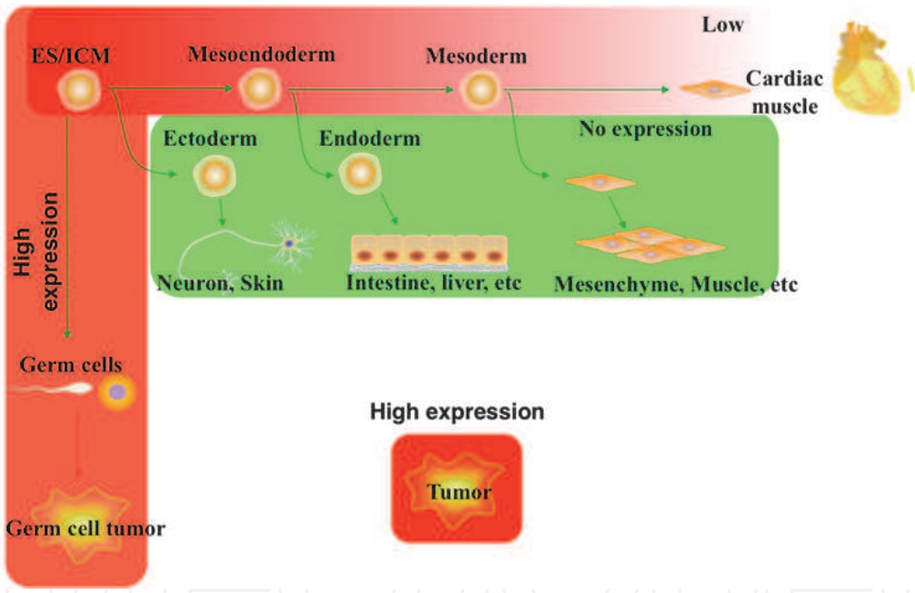

Fig. 4. Expression of Cripto-1 during embryogenesis and tumorigenesis.

Cripto-1 is highly expressed in undifferentiated embryonic stem cells and germ cells. Cripto-1 is important for mesoendoderm differentiation of ES cells and its expression is lost upon differentiation of ES cells toward the three germ cell layers. In the adult, Cripto-1 is re-expressed by tumor cells. Green color: no Cripto-1 expression, red color: Cripto-1 expression. ICM: Inner cell mass, ES: embryonic stem cells.

4.1 Cripto-1 oncogenic activities *in vitro* and *in vivo*

The first evidence of Cripto-1 oncogenic activity derives from studies demonstrating that Cripto-1 overexpression can induce *in vitro* transformation of a variety of cells, including NIH-3T3 fibroblasts and NOG-8 or CID9 mouse mammary epithelial cells (Ciardiello et al.,

1991; Ciccodicola et al., 1989; Niemeyer et al., 1998). Furthermore, Cripto-1 can enhance migration and invasion of a variety of normal mammary epithelial cells and human breast and cervical carcinoma cells, suggesting the involvement of Cripto-1 in tumor progression (Ebert et al., 2000; Normanno et al., 2004a; Wechselberger et al., 2001). Cripto-1 can also function as a potent angiogenic protein both *in vitro* and *in vivo*, enhancing the proliferation, migration and invasion of human umbilical vascular endothelial cells and stimulating their differentiation into vascular-like structures in matrigel. Likewise, overexpression of Cripto-1 promotes *in vivo* neovascularization of MCF-7 xenografts, supporting a role for Cripto-1 in modulating tumor angiogenesis (Bianco et al., 2005a). Since HIF-1α can enhance Cripto-1 expression by directly binding to the Cripto-1 promoter, it is possible that the hypoxic microenvironment within the growing tumor might enhance Cripto-1 expression, which in turn induces new vessel formation to sustain tumor growth (Bianco et al., 2009). Cripto-1 has also been directly implicated in mouse mammary tumor development (Sun et al., 2005; Wechselberger et al., 2005), as demonstrated by transgenic mouse models overexpressing the human Cripto-1 transgene in mouse mammary glands under the control of the MMTV or whey acidic protein (WAP) promoter (Sun et al., 2005; Wechselberger et al., 2005). The majority of nulliparous MMTV-Cripto-1 transgenic mice exhibited enhanced ductal branching, intraductal hyperplasias and hyperplastic alveolar nodules, and about 30-40% of aged multiparous female mice developed multifocal hyperplasias and papillary adenocarcinomas (Wechselberger et al., 2005). Interestingly, under a 2 years observation period, approximately 20% of female nulliparous or multiparous MMTV-Cripto-1 transgenic mice developed uterine leiomyosarcomas as compared to control mice. High levels of Cripto-1 transgene, phosphorylated forms of c-Src, AKT, glycogen synthase kinase-3β (GSK-3β) and dephosphorylated active-β-catenin were detected in the uterine tumors collected from MMTV-Cripto-1 transgenic mice (Strizzi et al., 2007), suggesting a role for Cripto-1 during uterine tumorigenesis either by activation of c-Src and Akt and/or via cross-talk with the canonical Wnt/β-catenin signaling pathway. Unlike the MMTV promoter, the WAP promoter is maximally active during mid-pregnancy and lactation. Approximately 50% of old nulliparous WAP-Cripto-1 mice developed multifocal intraductal hyperplasias, and more than 50% of multiparous WAP-Cripto-1 female mice developed multifocal mammary tumors of mixed histological subtypes (Sun et al., 2005). Histological analysis of the WAP-Cripto-1 mammary tumors revealed the presence of tumors containing glandular, papillary and undifferentiated carcinoma, as well as myoepithelioma and adeno-squamous carcinoma. Mammary tumors with mixed histology have also been described in MMTV-Wnt-1 transgenic mice, which have alterations in the canonical Wnt/β-catenin pathway. Interestingly, hyperactivation of a canonical Wnt/β-catenin pathway was detected in WAP-Cripto-1 mammary tumors, suggesting that the canonical Wnt/β-catenin pathway together with Cripto-1 might play an important role during mammary transformation *in vivo* (Miyoshi et al., 2002a; Miyoshi et al., 2002b; Sun et al., 2005). Finally, several studies have demonstrated that Cripto-1, in addition to function as an oncogene *in vitro* and in animal models, is overexpressed at the mRNA and protein level in a wide variety of human tumors, including colorectal, breast, gastric, pancreatic, ovarian, endometrium, nasopharynx and lung carcinomas, while very little or no expression is detected in normal adult tissues. Therefore, the selective expression of Cripto-1 in cancer cells suggests that it represents a promising target in cancer therapy. For an extensive review on Cripto-1 expression in human tumors see de Castro et al., 2010.

4.1.1 Intracellular signaling pathways activated by Cripto-1 during oncogenic transformation

While Cripto-1 functions mostly in a Nodal-dependent manner during embryogenesis, several studies have demonstrated that Cripto-1 induces cellular proliferation, motility, survival and EMT in a Nodal-independent fashion. Following binding to the GPI-linked heparan sulphate proteoglycan Glypican-1, Cripto-1 induces activation and phosphorylation of the cytoplasmic tyrosine kinase c-Src, which in turn activates mitogen-activated protein kinase (MAPK)/Phosphatydil inositol 3′ kinase (PI3K)/Akt signaling pathways (Figure 5) (Bianco et al., 2005b).

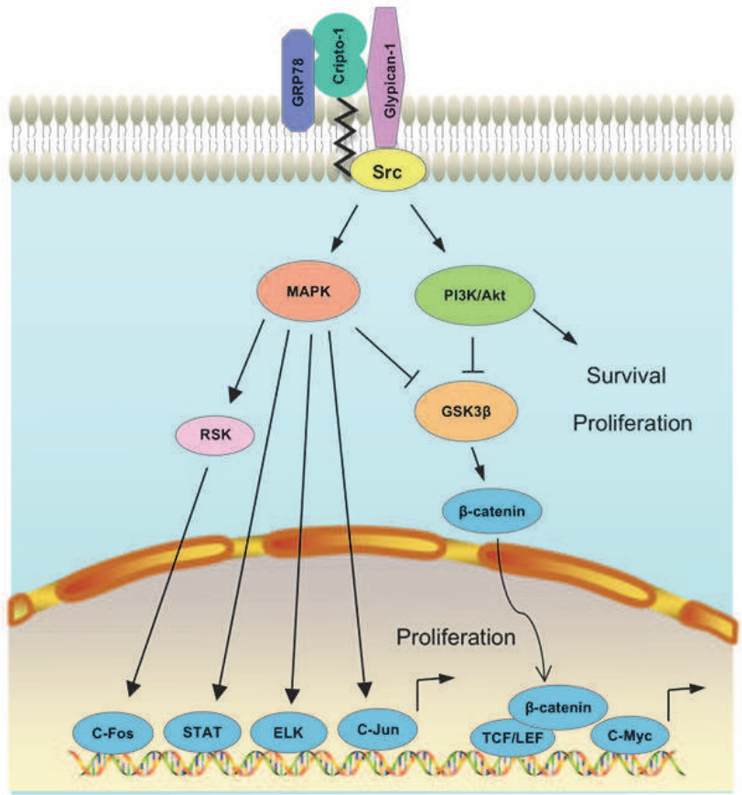

Fig. 5. Nodal-independent Cripto-1 signaling pathway.

Cripto-1 upon binding to Glypican-1 activates MAPK and Akt signaling pathways during tumor progression, enhancing cell proliferation and survival. MAPK and PI3K/Akt pathways can also inhibit GSK-3β leading to activation and stabilization of β-catenin. GRP78 can also enhance Cripto-1 activation of the MAPK/Akt signaling pathways. GRP78: glucose-regulated protein 78, MAPK: mitogen-activated protein kinase, PI3K: phosphatydil inositol 3′ kinase, GSK3β : glycogen synthase kinase 3β, TCF/LEF: T-cell factor/lymphoid enhancer factor, RSK: Ribosomal s6 kinase, STAT: signal transducers and activators of transcription.

Activation of the MAPK and PI3K/Akt signaling pathways by Cripto-1 is independent of Nodal and ALK4, since Cripto-1 can enhance phosphorylation of MAPK and Akt in cells lacking ALK4 and/or Nodal expression (Bianco et al., 2002). Furthermore, 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 (Bianco et al., 2003). The interaction between Cripto-1 and Gypican-1 occurs within lipid raft microdomains, regions of the cell membrane that are enriched in GPI-linked proteins (Bianco et al., 2003). Cripto-1 mitogenic signaling is also dependent upon binding to GRP78, an endoplasmic reticulum (ER) heat shock chaperone protein that is expressed on the cell surface of tumor cells (Shani et al., 2008). Indeed, disruption of the Cripto-1/GRP78 complex at the cell membrane interferes with Cripto-1 oncogenic activity *in vitro*, preventing Cripto-1 activation of MAPK/Akt signaling pathway (Figure 5). Interestingly, unlike Glypican-1, GRP78 is also important for Nodal-dependent Cripto-1 signaling, enhancing activation of the Smad-2/Smad-3 signaling pathway (Kelber et al., 2009). Furthermore, GRP78 enhanced Cripto-1-dependent activation of the c-Src/MAPK/Akt signaling pathways, increased cellular proliferation and reduced cell adhesion, as evidenced by a decrease in E-cadherin expression in breast cancer cells (Kelber et al., 2009).

5. Cripto-1 and Cancer Stem Cells

Cancer stem cells (CSCs), also known as tumor initiating cells, share characteristics associated with embryonic stem cells, specifically the ability to give rise to all cell types within a particular tumor tissue. CSCs were first identified in the hematopoietic system and later they have also been reported in solid cancers, including cancers of the breast, lung, prostate, colon, brain, head and neck, and pancreas (Bianco et al., 2010; de Castro et al., 2010). CSCs represent a distinct population of cancer cells with innate chemo- and radio-resistance and therefore are responsible of tumor relapse (Bianco et al., 2010; de Castro et al., 2010 as cited in Huntly and Gilliland 2010). Moreover, CSCs are capable to self-renew and regenerate the original phenotype of the tumor when implanted into immunodeficient mice (Visvader & Lindeman, 2008). Similarities between embryonic development and cell transformation during oncogenesis have led to the identification of common contributing pathways, suggesting that reactivation of developmental signaling pathways might drive cell transformation and tumor progression in adult tissues (Bianco et al., 2010). Cripto-1 is a typical example of a common gene shared by embryonic cells and cancer cells contributing to early embryogenesis and cancer progression. More importantly, Cripto-1 is enriched in a subpopulation of cancer cells with stem-like characteristics. For instance, Watanabe and collaborators (2010) described a heterogeneous Cripto-1 expression pattern in human embryonal carcinoma (EC) with segregation of these cells into two distinct populations portraying high and low Cripto-1 expression. EC cells are pluripotent stem cells derived from germ cell teratocarcinomas and they represent the malignant counterparts of human ES cells. Interestingly, these two subpopulations showed different gene expression profiles and different *in vitro* and *in vivo* tumorigenic capability. The Cripto-1 high subpopulation of EC cells formed tumor spheres in a serum-free suspension culture with an efficiency significantly higher than the Cripto-1 low expressing EC cells. Furthermore, the Cripto-1 high expressing EC cells were able to generate tumors larger in size and with a shorter tumor latency period compared with tumors derived from Cripto-1 low expressing EC cells when injected subcutaneously into nude mice. Finally, regulators of pluripotent ES cells, such as Activin/Nodal signaling and the transcription factors Nanog and Oct-4, positively regulated Cripto-1 expression in Cripto-1 high expressing EC cells, suggesting the existence of a core transcriptional regulatory network of pluripotent stem cell transcription factors that cross-regulate each other expression (Loh et al., 2006). However, Cripto-1 expression is

dispensable for pluripotent stem cell gene expression in EC cells, since siRNA-mediated knockdown of Cripto-1 has no effect on the expression of ES-related genes including Oct-4, Nanog, Sox-2 and Lefty. In another study, Cripto-1 has been exploited as a potential melanoma stem cells candidate marker in human malignant melanomas (Strizzi et al., 2008). Strizzi and colleagues, by FACS cell sorting based on Cripto-1 expression, isolated from a human melanoma cell line a subpopulation with stem-like characteristics. The Cripto-1 positive subpopulation of melanoma cells represented a relatively smaller size population with a more spindle-shaped morphology, and showed increased expression of the stem cell associated transcription factors, Oct-4 and Nanog, as compared to the parental melanoma cells. Finally, Cripto-1 and the stem cell markers Oct-4 and SUZ-12 were identified in a small subpopulation of stem-like cells in hormone-responsive and nonresponsive prostate tumor cell (Cocciadiferro et al., 2009). Altogether, these findings suggest that Cripto-1 might be useful in the identification of a subpopulation of cancer cells with stem like behavior that are resistant to conventional therapy and are therefore a major obstacle in the clinical treatment of cancer patients.

5.1 Cancer stem cells and EMT

During embryogenesis, tumor progression and metastasis, epithelial cells undergo dramatic morphological changes, acquiring mesenchymal properties in a process known as EMT. In the embryo, Cripto-1 is detected at high levels in epiblast cells undergoing EMT, which migrate through the primitive streak, eventually giving rise to the mesoderm and endoderm (Bianco et al., 2005b; Strizzi et al., 2005). In the tumor, the expression of EMT regulators at the tumor periphery is critical for tumor cells to acquire a mesenchymal phenotype that allow them to locally invade and escape from the primary tumor site, leading to the establishment of metastatic lesions (Micalizzi et al., 2010a). Cripto-1 is involved in tumor epithelial cells plasticity and may be an important EMT regulator together with Snail, Slug, Twist, and Six1 (Micalizzi et al., 2010b). It has been shown that mammary gland hyperplasias and tumors derived from MMTV-Cripto-1 transgenic mice express molecular markers and signaling molecules characteristics of EMT, suggesting that Cripto-1 might play an important role in facilitating migration and invasion of tumor cells (Strizzi et al., 2004). These findings might be significant since emerging evidence has suggested a link between stem cells and EMT (Hollier et al., 2009). In fact, EMT induction in immortalized human mammary epithelial cells resulted in the expression of stem cell markers and increased ability to form mammospheres *in vitro*, suggesting an important role for EMT in generating cancer stem-like cells in human breast tumors (Mani et al., 2008). Since Cripto-1 has been found to promote EMT in mouse mammary epithelial cells and mouse mammary tumors, contributing to a more aggressive mesenchymal phenotype, it is possible that Cripto-1 might support self-renewal, invasiveness and metastatic abilities of breast cancer stem-like cells through induction of an EMT program (Strizzi et al., 2004).

6. Cripto-1 as target for cancer therapy

High expression of Cripto-1 in human carcinomas and its enrichment in a stem-like cancer cell subpopulation strongly support Cripto-1 as a promising candidate for therapeutic intervention in cancer. Two different therapeutic approaches have been successfully used to impair Cripto-1 activity in cancer cells, including anti-Cripto-1 antisense (AS) oligonucleotides and neutralizing monoclonal antibodies (Adkins et al., 2003; Hu et al., 2007;

Normanno et al., 1996; Normanno et al., 2004b). For instance, a significant growth inhibition *in vitro* has been observed in ovarian, breast and colon cancer cells treated with anti-Cripto-1 AS oligonucleotides. A synergistic growth inhibitory effect was detected when anti-Cripto-1 AS oligonucleotides were combined together with oligonucleotides against TGF-α and amphiregulin (AREG), indicating that a variety of growth factors might cooperate in stimulating cancer cell proliferation (Casamassimi et al., 2000; De Luca et al., 2000; De Luca et al., 1999; Normanno et al., 2004b). Additionally, combination of anti-Cripto-1 AS oligonucleotides with anti-TGF-α and anti-AREG AS oligonucleotides was more effective *in vivo* as compared to single AS oligonucleotide administration, inhibiting the growth of colon cancer tumor xenografts in nude mice in a dose dependent manner (De Luca et al. 2000). Neutralizing antibodies that bind Cripto-1 have also been generated (Adkins et al., 2003; Hu & Xing, 2005). In particular, Adkins and colleagues of Biogen-Idec have generated a panel of monoclonal antibodies (mAbs) directed against a human recombinant Cripto-1 protein expressed as a human IgG1 Fc fusion protein (Adkins et al. 2003). The mAbs are capable to recognize different domains of the Cripto-1 protein, detecting its expression in human breast and colon tumor tissue samples and in human cancer cell lines. Among these anti-Cripto-1 mAbs, anti-CFC mAbs showed blocking activities *in vitro* and *in vivo* by interfering with the binding of Cripto-1 with ALK4 through the CFC domain. The anti-CFC blocking antibodies also blocked the interaction of Cripto-1 with Activin B (Adkins et al. 2003), restoring the growth inhibitory function of Activin B in tumor cells. Recently, among these anti-Cripto-1 mAbs, an N-terminal anti-Cripto-1 antibody has been selected to target Cripto-1 in human tumors, due to the higher binding affinity and range of reactivity of this antibody with tumor-derived Cripto-1 (Kelly et al., 2011). A humanized form of the anti-Cripto-1 N-terminal monoclonal antibody-conjugated to a DM4 toxin is currently been 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) (Bianco & Salomon, 2010). BIIB015 showed specificity for Cripto-1 *in vitro* and *in vivo* with a clinical response ranging between 50% of tumor inhibition to complete tumor regression in xenograft mouse models inoculated with lung (Calu-6), colon (CT-3), testicular (NCCIT) or breast (MDA-MB-231) human tumor cell lines (Kelly et al., 2011). In addition to mouse monoclonal antibodies against Cripto-1, anti-Cripto-1 rat monoclonal antibodies have also been generated. Rat anti-Cripto-1 monoclonal antibodies inhibited tumor cell growth and increased sensitivity of LS174T human colon cancer cells and MCF-7 human breast cancer cells to the cytotoxic effects of chemotherapeutic agents such as cisplatin, 5-Fluorouracil, carboplatin and epirubicin (Xing et al., 2004). Furthermore, a C13 rat monoclonal antibody inhibited cancer cell growth *in vivo* in LS174T tumor xenografts in SCID mice, inducing up to 80% growth inhibition of established tumors as compared to an untreated control groups. Interestingly, C4 and C13 rat monoclonal antibodies were also 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 (Hu et al., 2007).

6.1 Cripto-1 as a target for therapy in neurodegenerative and muscle degenerative diseases

Recent findings have demonstrated that Cripto-1 is a key player in the signaling pathway controlling neural induction in ES cells. Parisi and collaborators showed that Cripto-1

negatively regulated neuronal differentiation of ES cells (Parisi et al., 2003). Furthermore, disruption of Cripto-1 expression in mouse ES cells enhances neurogenesis and midbrain dopaminergic differentiation *in vitro* (Parish et al., 2005; Sonntag et al., 2005). These results suggest that blocking Cripto-1 expression and activity may provide a novel insight into the treatment of neurodegenerative diseases. Parkinson's disease is a progressive neurodegenerative disorder characterized by degeneration of 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 ES cells grafting that often results in uncontrolled proliferation and teratoma formation (Parish & Arenas, 2007). In a rat animal model of Parkinson's disease, mouse ES cells that are null for Cripto-1 expression (Cripto-1$^{-/-}$ ES cells), when grafted at low density into rats, differentiated into neuronal cells in the brain and were able to restore normal behavior without producing teratomas (Parish et al., 2005). Recently, a tetrameric tripeptide, which prevents Cripto-1/ALK4 interaction and interferes with Cripto-1 signaling was identified (Lonardo et al., 2010). The Cripto-1 blocking peptide was able to induce formation of neuroectoderm and increased midbrain dopaminergic neuron differentiation of mouse ES cells *in vitro* and *in vivo* (Lonardo et al., 2010). Moreover, mouse ES cells treated with Cripto-1 blocking peptide enhanced functional recovery and reduced tumor formation in Parkinsonian rats (Lonardo et al., 2010). Overexpression of Cripto-1 has also been observed in the cerebral cortical tissues of macaques that had been infected with a chimeric simian human immunodeficiency virus (SHIV) by cDNA array analysis (Stephens et al., 2006). SHIV enters the central nervous system (CNS) early after inoculation and cause encephalitis, characterized by transient meningitis and astrocytosis. Immunohistochemical analysis and RT-PCR confirmed widespread expression of Cripto-1 in the brains of SHIV-infected macaques. Although the functions of Cripto-1 in the neurons of the adult brain and the overexpression in the brains of SHIV-infected macaques are unclear, Cripto-1 may play a neuroinflammatory role and targeting Cripto-1 with various approaches, such as the tetrameric tripeptide described by Lonardo and colleagues, might inhibit progression of neurodegenerative disease in mammals. Cripto-1 may also be involved in degenerative muscle diseases, such as muscular dystrophy and spinal muscular atrophy. These diseases are characterized by skeletal muscle loss and few therapeutic approaches are available to restore the function of the lost muscle tissue (Tedesco et al., 2010). Cripto-1 is highly expressed in myoblast cells of regenerative muscles, whereas no expression of Cripto-1 is detected in normal muscle fibers. Furthermore, Cripto-1 induced a dose-dependent increase in proliferation and migration of myogenic precursor cells *in vitro* and enhanced skeletal muscle regeneration and angiogenesis after injury *in vivo* (Bianco & Salomon, 2010). These studies clearly suggest an involvement of Cripto-1 in neurodegenerative and muscle degenerative disease. Therefore, approaches that have been developed to target Cripto-1 in cancer might benefit also different diseases.

7. Conclusions

Critical signaling pathways are involved in modulating embryonic stem cell fate and behavior, maintaining a delicate balance between survival and self-renewal signals. Among these ES cell "signature" pathways, Cripto-1 is a critical gene that is used by ES cells. For instance, Cripto-1 is either a downstream target of ES transcription factors and/or signaling pathways or can modulate other ES cell signaling cascades (Fig. 3). Further, deregulation of

stem cell self-renewal is probably a requirement for the initiation and formation of CSCs and therefore embryonic stem cell signature genes are also involved in cancer formation. Cripto-1 is indeed an embryonic gene that is re-expressed in an aberrant spatial and temporal manner in a variety of human tumors. Recent evidence has clearly demonstrated that Cripto-1 is expressed by a subset of cancer cells with stem-like characteristics (Watanabe et al., 2010). CSCs are considered to be a major obstacle in the complete eradication of tumors due their innate resistance to conventional therapy and therefore identification of surface markers that might discriminate CSCs from the bulk population of tumor cells is under active investigation. Therefore, Cripto-1 targeting in human tumors might have a major breakthrough in cancer therapy. Several approaches have been used to target Cripto-1 in cancer cells *in vitro* and *in vivo*, from antisense oligonucleotides to blocking monoclonal antibodies (Bianco & Salomon, 2010). Translation of Cripto-1 research to a clinical setting has been recently achieved. In fact, a humanized anti-Cripto-1 antibody conjugated with a potent cytotoxin is currently being evaluated in a Phase I clinical study in cancer patients. In conclusion, a complete understanding of Cripto-1 signaling in stem cell biology offers a great promise for improving stem cell mediated regenerative therapy as well as cancer therapy.

8. References

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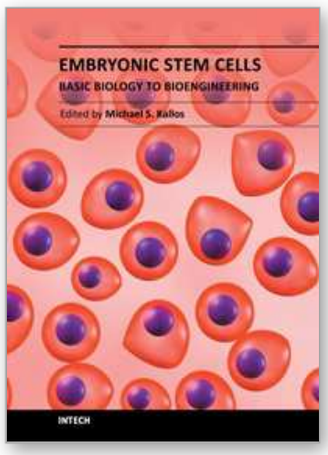

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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