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Cripto-1 Is a Cell Surface Marker for a Tumorigenic, Undifferentiated Subpopulation in Human Embryonal Carcinoma Cells

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Abstract

Deregulation of stem cells is associated with the generation and progression of malignant tumors. In addition, genes that are associated with early embryogenesis are frequently expressed in cancer. Cripto-1 (CR-1), a glycosylphosphatidylinositol-linked glycoprotein, is expressed during early embryogenesis and in various human carcinomas. We demonstrated that human embryonal carcinoma (EC) cells are heterogeneous for CR-1 expression and consist of two distinct subpopulations: a CR-1^{High} and a CR-1^{Low} population. By segregating CR-1^{High} and CR-1^{Low} populations of NTERA2/D1 EC cells by fluorescence-activated cell sorting, we demonstrated that CR-1^{High} cells were more tumorigenic than CR-1^{Low} cells by an in vitro tumor sphere assay and by in vivo xenograft formation. The CR-1^{High} population was enriched in mRNA expression for the pluripotent embryonic stem (ES) cell genes Oct4, Sox2, and Nanog. CR-1 expression in NTERA2/D1 cells was regulated by a Smad2/3-dependent autocrine loop, by the ES cell-related transcription factors Oct4/Nanog, and partially by the DNA methylation status of the promoter region. These results demonstrate that CR-1 expression is enriched in an undifferentiated, tumorigenic subpopulation and is regulated by key regulators of pluripotent stem cells.

Keywords

Cripto-1; Embryonal carcinoma; Nanog; Oct4; Pluripotent stem cells; Tumorigenicity

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Introduction

Pluripotent stem cells such as embryonic stem (ES) [1] or induced pluripotent stem (iPS) cells [2] have the capacity of self-renewal and differentiation into many cell types that are derived from all three germ layers. Discovery of direct reprogramming of somatic cells into iPS cells has opened up the possibility of patient-specific cell transplantation therapy to treat various diseases without having the ethical problems of human ES cells, which require human embryos. However, potential problems still exist, of which one is their tumorigenic capacity, that must be avoided on clinical application [3]. Therefore, understanding the mechanism of tumorigenicity of these pluripotent stem cells is important for potential regeneration therapy. In addition, this information may also contribute to elucidating the mechanism of the genesis of somatic tumors, in which early embryonic genes play essential roles [4,5].

Human embryonal carcinoma (EC) cells are derived from teratocarcinomas, which arise from transformed germ cells [6]. EC cells are stem cells from such tumors and are generally considered as a malignant counterpart of ES cells [7]. EC cells are known to be highly tumorigenic in mouse xenograft models and resulting teratomas typically contain differentiated tissues of all three germ cell layers. Interestingly, induction of differentiation by retinoic acid (RA) causes multilineage differentiation and reduced tumorigenicity of EC cells [8]. This suggests that stemness or the undifferentiated status accompanies tumorigenic capacity in EC cells.

Human *Cripto-1* (*CR-1*) is a member of the epidermal growth factor (EGF)-cripto-1/FRL-1/cryptic family of genes [9]. CR-1 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein [10,11], which acts as a coreceptor for the transforming growth factor- β (TGF- β) subfamily of ligands Nodal, growth differentiation factor-1 and -3 (GDF1 and GDF3). CR-1 performs multiple roles in the regulation of vertebrate development and in the progression of malignant tumors [9,12]. CR-1 was initially cloned from EC cells [13] and is known to be associated with the pluripotent status of mouse and human ES cells [9,14].

In the current study, we demonstrate that the expression of CR-1 in EC cell lines is heterogeneous and that these cells can segregate into two distinct populations: CR-1^{High} and CR-1^{Low}. These two populations showed different in vitro and in vivo tumorigenic capacities as well as gene expression profiles. In addition, we addressed the regulatory mechanisms of CR-1 expression in EC cells by examining Nodal/Activin signaling, expression of ES cell-related transcriptional factors Oct4 and Nanog, and DNA methylation status of the CR-1 promoter regions. These results suggest that CR-1 expression is associated with the undifferentiated, tumorigenic subpopulation of EC cells.

Materials and Methods

Cell Culture

NTERA2/D1 cells (American Type Culture Collection [ATCC], Manassas, VA, <http://www.atcc.org>) were maintained in McCoy's 5A medium (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 15% fetal bovine serum (FBS), 2-mM glutamine, and 1% penicillin and streptomycin. NCCIT (ATCC) and 293T (ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2-mM glutamine, and 1% penicillin and streptomycin. Cells under 40 passages were used unless otherwise specified. In some experiments, cells were cultured in serum-free OPTI-MEM (Invitrogen) as indicated.

Reagents

Recombinant human Activin B, human Nodal, human Lefty A, and human Follistatin were purchased from R&D systems (Minneapolis, MN, <http://www.rndsystems.com>). All trans-RA and SB-431542 were purchased from Sigma-Aldrich (St. Louis, MO, <http://www.sigmaaldrich.com>).

Immunofluorescence

After washing with phosphate-buffered saline (PBS), NTERA2/D1 cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. For costaining with phosphorylated Smad2 (pSmad2), cells were permeabilized with 0.2% Triton X-100 for 2 minutes at room temperature. Membrane staining of CR-1 was performed without permeabilization. After blocking with 20% normal goat serum and 2% bovine serum albumin in PBS, CR-1 was detected with 5 μ g/ml of anti-hCR1 monoclonal antibody (mAb) (MAB2771, R&D systems) and Alexa Fluor 488-conjugated secondary antibody (Invitrogen). pSmad2 was detected with anti-pSmad2 rabbit mAb (Cell Signaling, Denver, MA, <http://www.cellsignal.com>) and Alexa Fluor 564-conjugated secondary antibody (Invitrogen). Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used as a mounting medium.

Immunohistochemistry

Tumor tissues or cell pellets were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C and were paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E) for subsequent histological analysis. For immunohistochemistry, tissues cut in 5- μ m sections were deparaffinized in xylene and rehydrated in a series of graded ethanol. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 minutes. Antigen retrieval was performed by autoclaving slides in citrate buffer (pH 6.0). CR-1 protein was stained with mouse anti-CR-1 mAb (B3F6, Biogen Idec, Cambridge, MA, <http://www.biogenidec.com/>) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, <http://vectorlabs.com>) following the manufacturer's instructions. Sections were counterstained with hematoxylin.

Fluorescence-Activated Cell Sorting Analysis, Live-Cell Sorting, and Clone Generation

NTERA2/D1 cells grown to 70–80% confluency were collected with enzyme-free cell dissociation buffer (Invitrogen). After washing with ice-cold fluorescence-activated cell sorting (FACS) buffer (PBS with 0.1% bovine serum albumin), cells were incubated for 30 minutes with phycoerythrin-conjugated anti-hCR1 mAb (R&D systems). Oct4 was stained with allophycocyanin-conjugated anti-Oct4 mAb (R&D systems) after permeabilization with SAP buffer (0.1% saponin and 0.05% NaN₃ in Hanks' balanced salt solution [HBSS]). The cells were pelleted, resuspended in 500 μ l of ice-cold FACS buffer, and analyzed using a FACS-can instrument (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>). Dead cells were excluded using viability dye 7-Aminoactinomycin D. For live-cell sorting, ice-cold FACS buffer with 100 units per milliliter penicillin-streptomycin and 1 μ g/ml amphotericin B was used for staining and washing. Cell sorting was performed on a BD FACSaria equipped with FACS DiVa6.0 software operating at low pressure (20 psi) using a 100- μ m nozzle. Cell clusters and doublets were electronically gated out. Cells were routinely double sorted and postsort analysis typically indicated purities of >98% with minimal cell death (<10%). For clone generation, single cells were deposited into 96-well plates by automatic cell deposition unit and expanded. Only wells confirmed to contain a single cell after sorting (determined microscopically) were evaluated.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted using the RNA mini-extraction kit (Qiagen, Valencia, CA, <http://www1.qiagen.com>) according to the manufacturer's instruction. Complementary DNA (cDNA) was prepared from 1- μ g total RNA using Retroscript kit (Ambion, Austin, TX, <http://www.ambion.com>). Quantitative polymerase chain reaction (PCR) was performed using a MX3000P PCR and Brilliant SYBR Green QPCR master mix (Stratagene, La Jolla, CA, <http://www.stratagene.com>). Comparative analysis was performed between the genes of interest and the house keeping gene GAPDH. The sequences of the primer sets are shown in Supporting Information Table 1.

Tumor Sphere Assay

After live-cell sorting, 5×10^4 NTERA2/D1 cells were seeded into 24-well Ultra Low Attachment plates (Corning, Corning, NY) in DMEM/F12 (Invitrogen) supplemented with 20-ng/ml EGF, 40-ng/ml fibroblast growth factor-2, and 4-ng/ml heparin. Cells were cultured for 10 days at 37°C in 5% CO₂ and 100% humidity without replacing the culture medium. Tumor spheres larger than 50 μ m in diameter were counted. Counting was performed in a blinded manner, and three-independent experiments were performed.

In Vivo Xenograft Assay

In vivo tumorigenicity was assessed by both frequency and latency of tumor formation in 8-week-old athymic NCr-nu/nu mice obtained from the National Cancer Institute colony (Animal Production Program, Frederick, MD, http://web.ncifcrf.gov/research/animal_production_program/). All animal experiments were conducted in accord with accepted standards of humane animal care and approved by the Animal Care and Use Committee at the National Institutes of Health. Freshly sorted cells were suspended in a F12 (Invitrogen)/Matrigel (high concentration, BD Biosciences) mixture (4:1) and injected subcutaneously into the dorsal region in a 50 μ l volume. Mice were anesthetized by an i.p. injection of ketamine/xylazine (750 and 50 mg/kg body weight, respectively) in 200- μ l HBSS before injection of cell suspension. Tumor size was measured weekly using a caliper. Once a xenograft reached 1.0 cm in diameter, the experiment was terminated. In mice devoid of frank tumors, H&E stained sections of injection sites were examined for the presence of micro- and macroscopic lesions.

Western Blotting

Proteins were separated on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Invitrogen). CR-1, Oct4, and Nanog were detected with mouse anti-CR-1 B3F6 mAb (1:3,000), goat anti-Oct4 polyclonal antibody (pAb) (1:1,000, R&D systems), and goat anti-Nanog pAb (1:1,000, R&D systems), respectively. μ -Actin was used for an internal standard using horse radish peroxidase-conjugated anti- μ -actin mAb (Sigma-Aldrich).

RNA Interference

ON-TARGET plus SMART pool small interfering RNAs (siR-NAs) against OCT3/4, NANOG, and control pool were purchased from Dharmacon (Chicago, IL, <http://www.dharmacon.com>). Subconfluent NTERA2/D1 cells were transfected with 20- μ M siRNA pools using Lipofectamin 2000 (Invitrogen). Sequences for siR-NAs against CR-1 are provided in Supporting Information Table 1. For transfection, Amaxa nucleofections were performed with 2×10^6 subconfluent NTERA2/D1 cells in L kit (Lonza, Basel, Switzerland, <http://www.lonza.com>) containing 1 μ M of each siRNA or 0.5 μ g green fluorescent protein (GFP) control vector and plated into six-well plates. Lentiviral pLKO.1-short hairpin RNA (shRNA) clones were purchased from Open Biosystems (Huntsville, AL,

<http://openbiosystems.com>). The hairpin sequences of targeting shRNA are provided in Supporting Information Table 1.

Dual Luciferase Assay

Full-length CR-1 promoter (4 kb, pCR-1-Luc) luciferase reporter was previously described [15]. Oct4, Sox2, and Nanog cDNAs were cloned from NTERA2/D1 cells and were subcloned into pEF6/V5-His TOPO TA expression vector (Invitrogen). 293T or NTERA2/D1 cells that had been plated in 24-well culture plates were transfected with an optimized amount of expression vectors (pCR-1-Luc, 200–500 nanograms per well; TK-renilla, 5–50 nanograms per well; varying concentrations of Oct4, Sox2, or Nanog expression vectors, 100–200 nanograms per well). pEF6/V5-His empty vector was added to adjust for total amount of DNA. After 24 hours of transfection, dual luciferase assays were carried out using a kit provided by Promega (Madison, WI, <http://www.promega.com>) according to the manufacturer's instructions.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, <http://www.activemotif.com>) according to the manufacturer's instruction. After crosslinking with 1% formaldehyde, chromatin from 1×10^7 NTERA2/D1 cells was sheared by sonication with optimized condition so that the resulting chromatin yields ~200–800-bp fragments. Immunoprecipitations were performed with anti-Oct4 rabbit pAbs (H-65, Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>), anti-Nanog goat pAb (AF1997, R&D systems) and anti-RNA polymerase II (RNA polII) mouse mAb (Active Motif). Normal IgGs were used for negative controls. After reverse-crosslinking and proteinase K treatment, precipitated chromatin was analyzed by quantitative PCR as described above. GAPDH and Nanog primer sets were used as negative and positive controls, respectively. The sequences of the primer sets are shown in Supporting Information Table 1.

Bisulfite Pyrosequencing

Bisulfite reaction was performed using the Epi-Tect Bisulfite kit (Qiagen) according to the manufacturer's instruction. Two micrograms of genomic DNA was used for each reaction. After PCR amplification using primer sets described in Supporting Information Table 1, CpG methylation was quantified by pyrosequencing with the PSQ96HS system (Pyrosequencing AB, Uppsala, Sweden).

Statistical Analysis

Student's *t*-test was used to determine the statistical significance of the quantitative results. Results with a *p* value <0.05 were considered statistically significant.

RESULTS

Heterogeneous Expression of CR-1 in EC Cells

To address the relationship of CR-1 expression in EC cells with respect to their differentiation status or tumorigenic capacity, we assessed CR-1 expression in two human EC cell lines, NTERA2/D1 and NCCIT. Immunostaining revealed a heterogeneous expression pattern of CR-1 in both cell lines (Fig. 1A and 1B). Wild-type and CR-1 stably transfected CHO cells were used as negative and positive controls, respectively. Since CR-1 is expressed on the cell surface as a GPI-anchored protein [10], its expression can be evaluated by FACS after live-cell staining. As a result of FACS analysis, NTERA2/D1 and NCCIT cells were clearly segregated into two subpopulations: CR-1^{High} and CR-1^{Low} (Fig.

1C). The percentage of cells in the CR-1^{High} subpopulation varied depending on the culture conditions. For example, the CR-1^{High} population in NTERA2/D1 cells was $60.6 \pm 16.1\%$ when the cells were cultured in regular growth medium supplemented with 15% serum and this ratio was decreased to $30.2 \pm 4.1\%$ after 3-day culture in serum-free medium (Fig. 1D). This downregulation of CR-1 expression was accompanied by a downregulation in the expression of the pluripotency-related factors Oct4 or Nanog (Fig. 1E). There was no significant difference when the cells were plated at different densities. However, when the cells were cultured for more than 5 days and were overconfluent, the CR-1^{High} population was significantly decreased to $\times 20$ –40% even in the presence of serum (data not shown). In accordance with the fact that CR-1 functions as a Nodal coreceptor, which activates an intracellular Smad2/3 signaling pathway, CR-1^{High} cells exhibited higher levels of nuclear pSmad2 (Fig. 1F and 1G).

Characterization of CR-1^{High} and CR-1^{Low} Subpopulations in EC Cells

We then attempted to characterize the biological differences that might exist between CR-1^{High} and CR-1^{Low} cells after live-cell sorting (Fig. 2A). After overnight culture on plastic, the two subpopulations showed different morphological phenotypes. The CR-1^{High} cells were relatively small and round, whereas the CR-1^{Low} cells showed a more heterogeneous morphology in which some cells are flat-shaped and some spindle-shaped (Fig. 2B). To address whether these morphological differences were accompanied by an alteration in gene expression, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to assess mRNA expression of germ layer lineage markers and pluripotency-related genes (Fig. 2C). Expression of CR-1 mRNA was ~ 20 -fold higher in the CR-1^{High} subpopulation, confirming that cell surface expression of the CR-1 protein was correlated with the level of mRNA expression. Markers associated with ES cells (*Oct4*, *Sox2*, *Nanog*, *Lefty*, *c-Kit*, *GDF3*, and *Nodal*) were two- to fourfold higher in CR-1^{High} cells. Endodermal markers (*BMP2*, *GATA4*, and *AFP*) were higher in CR-1^{Low} cells except for *FOXA2*. All mesodermal markers tested (*MSX1*, *T*, and *Vim*) were higher in CR-1^{Low} cells. As knockdown of CR-1 in mouse ES cells can induce neuronal differentiation [16], the neuroectodermal markers (*NEUROD1* and *GFAP*) were slightly higher in CR-1^{Low} cells. Costaining of CR-1 and Oct4 revealed that Oct4 was preferentially expressed in the CR-1^{High} subpopulation of NTERA2/D1 cells (Fig. 2D). The correlation of CR-1 and Oct4 expression was also observed in the NTERA2/D1 teratocarcinoma xenografts (Fig. 2E). Collectively, these results suggested that the CR-1^{High} subpopulation was enriched for an undifferentiated subpopulation of EC cells.

We did not observe any significant difference between these two populations in anchorage-dependent cell proliferation in the presence of serum, as assessed by an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or by monitoring cell number (data not shown). Thus, a tumor sphere forming assay in a serum-free suspension culture was used as an in vitro model to assess clonogenic capacity (Fig. 2F). The CR-1^{High} subpopulation showed a significantly higher ability than the CR-1^{Low} subpopulation to form tumor spheres (Fig. 2F). To determine whether this phenotype could be recapitulated in vivo, we performed subcutaneous transplantation of sorted NTERA2/D1 cells into nude mice. Even though we did not observe any significant difference in tumor incidence between these two populations with serial dilutions ranging from 1,000 to 500,000 cells per injection, there was a significant difference in the tumor-growth rate and tumor latency period between the CR-1^{High} and CR-1^{Low} subpopulations (Fig. 2G and 2H). CR-1^{High} cells formed tumors that were two- to fourfold larger than tumors that arose from CR-1^{Low} cells. In addition, the tumor-free survival rate was reduced by nearly twofold in the CR-1^{High} versus CR-1^{Low} subpopulations (50% tumor-free time: ~ 28 vs. ~ 50 days, respectively). Tumors from both populations showed similar histology of teratocarcinomas characterized by structures

derived from different germ cell layers and CR-1 expression was detected in both CR-1^{High} and CR-1^{Low} tumors (Fig. 2I). Disruption of the cell surface antigenicity of CR-1 after enzymatic digestion of the tumors precluded the application of FACS analysis on these tumors. As an alternative approach to quantify the level of CR-1 expression in these tumors, we performed Western blot analysis (Fig. 2J). Interestingly, although tumors from CR-1^{High} cells tended to express a higher level of CR-1 protein, some tumors expressed low levels of CR-1 protein whereas some tumors arising from CR-1^{Low} cells also regained CR-1 expression. Two reasons may possibly account for the recovery of CR-1 expression in the CR-1^{Low} cell-derived tumors. First, de novo generation of a CR-1^{High} population from CR-1^{Low} cells may occur. Alternatively, there might have been contamination of the CR-1^{High} cells in the initial CR-1^{Low} subpopulation. The latter possibility was unlikely since our sorting was quite stringent (>98% purity). To further address this question, clones from single cells were propagated from CR-1^{High} and CR-1^{Low} cells. In accordance with the clonogenic tumorsphere assay, cloning efficiently was higher for CR-1^{High} cells with 26 of 68 wells (38%) forming clones versus 5 of 61 wells (8%) forming clones for CR-1^{Low} cells. FACS analysis of these established clones revealed that most of the single CR-1^{Low} cell-derived clones produced heterogeneous progenies of CR-1^{High} and CR-1^{Low} phenotype, suggesting that the CR-1^{High} and CR-1^{Low} phenotypes are reversible (Fig. 2K). This possibility is also supported by the appearance of tumors, which possess low CR-1 protein expression that were derived from a population of CR-1^{High} cells, suggesting a reduction in CR-1 expression may occur in potentially more differentiated tumors.

Regulation of CR-1 Expression by Nodal/Activin Signaling and Pluripotent Stem Cell Transcription Factors

We have previously demonstrated that a TGF- β -Smad2/3 signaling positively regulates CR-1 expression in EC and colon cancer cells [17]. Therefore, we ascertained whether Activin or Nodal stimulation could enhance the percentage of cells in the CR-1^{High} subpopulation. Growth of NTERA2/D1 cells in serum-free medium resulted in ~50% reduction in the number of cells in the CR-1^{High} subpopulation (Fig. 1D). Stimulation with Activin B or Nodal significantly enhanced the number of cells in the CR-1^{High} subpopulation (Fig. 3A). SB-431542, which is a pharmacological inhibitor of the TGF- β type I receptors Alk4/5/7, almost completely eliminated the CR-1^{High} subpopulation (Fig. 3A). However, treatment of NTERA2/D1 cells with Follistatin or Lefty, which are endogenous inhibitors of Activin or Nodal, respectively, did not cause a significant downregulation of CR-1 expression (data not shown), suggesting a presence of autocrine signaling of an Alk4/5/7 ligand(s) other than Activin or Nodal in EC cells. The NTERA2/D1 CR-1^{High} subpopulation was also significantly depleted after BMP4-induced differentiation, which negatively regulates CR-1 expression in EC cells as previously described [17] (data not shown).

We then assessed whether pluripotent stem cell-related transcription factors such as Oct4 or Nanog might regulate CR-1 expression in EC cells. To this end, we used siRNA-mediated knockdown of Oct4 and Nanog (Fig. 3B). Knockdown of Oct4 or Nanog significantly suppressed the percentage of NTERA2/D1 cells in the CR-1^{High} subpopulation (Fig. 3C, si-control; $55.3 \pm 6.9\%$, si-Oct4; $20.8 \pm 14.6\%$, si-Nanog; $26.4 \pm 6.1\%$) in agreement with our previous findings that Nanog can regulate human and mouse CR-1/Cr-1 expression in EC cells [18] and mammary epithelial cells [19]. siRNA-mediated knockdown of Oct4 or Nanog also abolished the effect of Activin B or Nodal in enhancing the number of EC cells in the CR-1^{High} subpopulation in the absence of serum (Fig. 3D), suggesting that Oct4 and Nanog may act as downstream regulators of Nodal/Activin signaling. qRT-PCR confirmed the knockdown of Oct4 or Nanog mRNA expression after treatment of the NTERA2/D1 cells with si-Oct4 or si-Nanog, respectively (Fig. 3E). Importantly, Oct4 knockdown suppressed

Nanog mRNA expression and Nanog knockdown reciprocally impaired Oct4 mRNA expression. This result is in accord with the existence of a core transcriptional regulatory network of these pluripotent stem cell transcription factors that cross-regulate each others' expression [20]. Knockdown of Oct4 or Nanog also suppressed Activin B-induced mRNA upregulation of CR-1, Oct4, and Nanog, confirming the results described above (Fig. 3E).

CR-1 Is a Direct Target of Pluripotent Stem Cell Transcription Factors

These data suggested that CR-1 expression is positively regulated by the pluripotent stem cell transcription factors Oct4 and Nanog. Therefore, we analyzed the CR-1 promoter to ascertain whether these transcription factors can directly regulate CR-1 expression. The 4-kb promoter luciferase construct of human CR-1 [15] was transfected into 293T cells, which do not express endogenous Oct4 or Nanog (Fig. 4A). There was a slight but significant increase in the CR-1 promoter activity by overexpressing Nanog, or combination of Oct4 and Sox2 or all three factors compared with the empty vector control. However, the induction was subtle (approximately twofold), suggesting the presence of additional coactivators, which may be present in EC cells but absent in 293T cells or the presence of repressors, which are suppressed in EC cells but are expressed in 293T cells. Nevertheless, the CR-1 promoter activity in NTERA2/D1 cells was significantly suppressed by the siRNA-knockdown of Oct4 or Nanog (Fig. 4B), which confirmed the previous results (Fig. 3). We then performed ChIP analyses of the CR-1 promoter regions. Based on the transcriptional start site (TSS) of the human CR-1 gene, which we identified by 5'-rapid amplification of cDNA ends (RACE) of NTERA2/D1 cells at 255-bp upstream of the translational start site (K.W. and D.S.S., unpublished data), we designed 11 different PCR primer sets for ChIP analysis of the CR-1 promoter ranging from position -3,755 to +405 (Fig. 4C). ChIP analysis revealed that Oct4 and Nanog proteins bind to a region within ~1 kb of the TSS, corresponding to the previous findings by global ChIP-on-chip analysis, which showed that Oct4 and Nanog bind to the proximal region of the CR-1 promoter (~0.5 kb from TSS) in human ES cells [20]. To ascertain the binding of Oct4 or Nanog to the CR-1 promoter region, we performed ChIP assays with NTERA2/D1 cells before and after RA treatment using the Nanog and GAPDH promoter as controls. Oct4 and Nanog bound to Nanog promoter and CR-1 proximal promoter (I) but not to GAPDH promoter or CR-1 distal promoter (A) (Fig. 4D and 4E). The binding of Oct4 and Nanog proteins to the Nanog or CR-1 promoters was decreased after RA treatment (Fig. 4D and 4E). ChIP assay for RNA polII was also performed. As shown in Figure 4F, RNA polII was bound to the promoter region of Nanog, CR-1 (proximal only) and GAPDH, indicating active transcription of these factors. RA treatment decreased the association of RNA polII with the Nanog and CR-1 proximal promoters, whereas RNA polII binding was maintained for the GAPDH promoter after RA treatment. This RNA polII binding pattern was compatible with the mRNA expression level of these genes before and after RA treatment [21].

CR-1 Expression Is Partially Regulated by DNA Methylation Status of Its Promoter Region

Epigenetic remodeling is known to be important in determining the status of pluripotent stem cells [22,23]. Therefore, we compared the DNA methylation status of the CR-1 promoter between the CR-1^{High} and CR-1^{Low} subpopulations. We analyzed the CR-1 promoter methylation in NTERA2/D1 cells by pyrosequencing after bisulfite PCR in two different regions: a relatively CpG-rich region at ~0.5–0.8-kb upstream of TSS and a region surrounding the TSS (Fig. 5A). RA treatment strongly induced DNA methylation in both regions in a time-dependent fashion (Fig. 5B). Analysis of the DNA methylation status of the CR-1^{High} and CR-1^{Low} subpopulations revealed a clear difference between the two regions (Fig. 5C). There was no difference in the methylation pattern in the TSS region between the CR-1^{High} and CR-1^{Low} subpopulations, whereas CR-1^{Low} cells showed higher DNA methylation status than CR-1^{High} cells in the upstream CpG-rich region. This suggests

that differences in the regulation of DNA methylation exists in RA-induced differentiation and in spontaneous CR-1^{High} and CR-1^{Low} subpopulations of EC cells within these two promoter regions and that heterogeneous expression of CR-1 in EC cells may be partially regulated by DNA methylation of the promoter.

CR-1 Expression Is Dispensable for the Pluripotent Stem Cell Gene Expression

We then assessed whether CR-1 expression is functionally related to the undifferentiated status of EC cells. To this end, we used siRNA to knockdown CR-1 mRNA and protein expression (Fig. 6A). siRNA-mediated knockdown of CR-1 did not affect the expression of ES-related genes including Oct4, Nanog, Sox2, and Lefty (Fig. 6B). We confirmed these results with lentiviral shRNAs against CR-1 (data not shown). In addition, expression of Oct4, Nanog, and Sox2 was also retained in mouse Cr-1-null ES cells or Cr-1-null F9 EC cells compared with their wild-type counterparts (data not shown). In contrast, when NTERA2/D1 cells were treated with 10 μ M SB-431542 for 48 hours, expression of CR-1, Oct4, Nanog, and Lefty was markedly decreased (Fig. 6C). A downregulation in the expression of CR-1, Oct4, and Nanog was also observed after treatment with RA. In contrast, Lefty expression was strongly induced by RA treatment as previously described [24]. These data collectively suggest the presence of a CR-1-independent Activin/Nodal/TGF- β -Smad2/3 autoregulatory mechanism, which is essential to maintain pluripotent stem cell-related gene expression in human EC cells.

Finally, we tested whether CR-1 has any functional role in tumorigenesis. To this end, we generated two independent lentiviral shRNA constructs against CR-1 to achieve stable knockdown of CR-1. Even though knockdown of CR-1 protein or mRNA by these lentiviral shRNA in NTERA2/D1 cells was sufficient (>70–80%) and there was a significant suppression of cell growth in vitro as previously reported [25], we failed to observe any significant effects of CR-1 shRNA compared with control shRNA on in vivo tumor growth, latency period, or tumor-initiation capacity assessed by subcutaneously transplanting different dilutions of NTERA2/D1 cells (data not shown). In addition, there was no difference in expression of pluripotency markers such as Oct4 or Nanog between control and CR-1 shRNAs, confirming the result from transient siRNA experiment (Fig. 6 and data not shown). Similar results of in vivo tumor transplants were observed with wild-type and Cr-1-null F9 mouse EC cells.

Discussion

In this study, we demonstrated that human EC cells are heterogeneous in CR-1 expression. A CR-1^{High} subpopulation could be isolated from the parental EC cell population. The CR-1^{High} EC cells exhibited undifferentiated characteristics of stem cells and showed a higher degree of tumorigenicity as compared with the CR-1^{Low} subpopulation. We also demonstrated that CR-1 expression was combinatorially regulated by a Smad2/3-dependent endogenous signaling pathway that can be activated by Activin or Nodal, by the ES cell-related transcription factors Nanog and Oct4 and partially by the degree of DNA methylation. These results suggest a potential model in which the CR-1 expression is positively regulated in a more undifferentiated, tumorigenic population (Fig. 7).

The heterogeneity of CR-1 expression in cultured EC cells might be due to the plasticity of EC cells, because EC cells exhibit the ability to spontaneously differentiate into various types of cells similar to ES cells [6] and like ES cells maintain a heterogeneous expression of pluripotency genes such as Oct4 and Nanog [26–29]. CR-1^{High} cells were enriched for the expression of undifferentiation markers and CR-1^{Low} cells expressed higher levels of germ layer lineage commitment markers. The fact that the CR-1^{Low} population was increased after confluency or after BMP-induced differentiation (data not shown) strongly suggests

that the level of CR-1 expression is inversely correlated with cell differentiation in vitro. On the other hand, we observed that CR-1^{High} cells can be generated from the CR-1^{Low} population over time both in an in vivo xenograft assay and in an in vitro clonal analysis. We can not fully distinguish whether these cells represent undifferentiated EC cells or cells that are derived from cardiac mesoderm progenitors that express CR-1 [30]. However, since ES cells are dynamically interchangeable among different ground states [29,31], it is possible that self-renewal, differentiation, and de-differentiation of EC cells are dynamically occurring simultaneously in the same culture, which can contribute to the heterogeneity of CR-1 expression.

One mechanism for maintenance of pluripotency in ES cells is the activation of a transcriptional self-regulatory circuit composed of the ES cell-related transcription factors Oct4, Sox2, and Nanog, which can also be reciprocally regulated [20,32–34]. The similarity of ES cells and EC cells in terms of pluripotency suggests that EC cells may also be maintained by a similar self- and cross-regulatory circuit of transcription factors. In the current study, we have demonstrated that CR-1 expression is directly regulated by Oct4 and Nanog. This result is consistent with data from a series of genome-wide ChIP analyses [20,32–34], which have shown the presence of Oct4 and Nanog binding sites within the CR-1 promoter region. This suggests that a self-regulatory circuit is activated in the CR-1^{High} subpopulation of EC cells, whereas the CR-1^{Low} subpopulation may arise when this self-regulatory transcriptional circuit is suppressed by either loss of expression of Oct4 and Nanog or DNA methylation. In this respect, the CR-1^{High} subpopulation in EC cells can be maintained by the activation of an autocrine Smad2/3-dependent signaling pathway. Since Activin/Nodal signaling through Smad2/3 can directly activate Oct4 or Nanog expression [35,36] and since these genes are essential for the maintenance of the pluripotency of human ES cells [37,38] or mouse epiblast-derived pluripotent stem cells [39], it is likely that a Smad2/3-dependent signaling pathway involving Activin, Nodal, or other Alk4/5/7 ligands such as GDF1 or GDF3 also performs an important role in maintaining a pluripotent and tumorigenic subpopulation in human EC cells. In fact, pharmacological inhibition of endogenous Smad2/3 signaling strongly suppressed the expression of these ES cell-related factors. Moreover, because CR-1 is a coreceptor of Nodal and GDF1/GDF3 signaling, positive regulation of CR-1 expression possibly by GDF1 or GDF3 also indicates the presence of a positive feedback regulatory network of this signaling pathway. However, the expression of Oct4 or Nanog was not significantly impaired after siRNA-mediated knockdown of CR-1 in EC cells, suggesting that endogenous Activin, TGF- β , or CR-1-independent signaling may be involved in the maintenance of Oct4 and Nanog expression in the CR-1^{High} subpopulation.

It has been suggested that tumorigenicity is related to the expression of essential regulators of pluripotency, such as Oct4 and Nanog, not only in germ cell tumors [40] but also in somatic cancers [41–45]. A recent study has demonstrated the expression of CR-1 in a small subset of stem-like cells in malignant melanoma and prostate carcinoma cells [45,46]. Therefore, CR-1^{High} cells could be a potential therapeutic target in various types of malignant tumors. Even though our data suggest that CR-1 is functionally redundant to maintain the ES cell-related gene expression or to support tumor growth in vivo, a previous study has shown that CR-1 functions as a mitogen in NTERA2/D1 cells [25] and that a blocking mAb against CR-1 can suppress growth of NCCIT EC cells as well as colon carcinoma in vivo [47]. This discrepancy between the current result and previous reports may be explained by higher specificity of the shRNAs technology compared with blocking antibodies or antisense oligo-DNAs or probably by some compensation and escape mechanism after a period of stable knockdown of CR-1 expression in which differentiated CR-1^{Low} cells can give rise to an undifferentiated CR-1^{High} subpopulation and thereby maintain tumor growth in vivo. Nevertheless, our data suggest that an undifferentiated

subpopulation of EC cells, which can be defined by high cell surface CR-1 expression is responsible for the initiation and early and rapid growth of transplanted EC cells. CR-1 is a potential target for drug delivery to a subpopulation of tumor cells because CR-1, which is a GPI-anchored protein, is expressed on the surface of undifferentiated tumor cells. In this regard, our group has developed a drug delivery system that targets CR-1 expressing tumor cells using humanized anti-CR-1 specific antibodies conjugated with antitumor drugs [47]. These agents are now in a phase I clinical trial. Finally, cell surface CR-1 expression could be utilized for exclusion of a tumorigenic subpopulation of ES/iPS-derived cells for regenerative cell therapy.

Conclusion

In this study, we demonstrated, by detailed promoter analysis with ChIP assay and by siRNA knockdown experiments, that CR-1, a GPI-anchored membrane protein, is a direct target of the pluripotent stem cell transcription factors Oct4 and Nanog in EC cells. Human EC cells are heterogeneous for CR-1 expression and a CR-1^{High} subpopulation of EC cells exhibited a more undifferentiated phenotype such as high expression of ES cell-related genes and showed a higher tumorigenic capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Heterogeneous expression of CR-1 in cultured EC cells. (A): Immunocytochemistry of NCCIT cells. CHO-WT cells and CR-1 stable transfectants (CHO-CR-1) were used as negative and positive controls, respectively. Scale bar = 50 μ m. (B): Immunocytochemistry of NTERA2/D1 cells. Cells were fixed and stained with anti-CR-1 mAb without permeabilization. Nuclei were counterstained with DAPI. Arrowheads indicate the cells that highly express CR-1 protein on the cell surface. Normal mouse IgG was used as a negative control. Scale bar 10 = μ m. (C): Fluorescence-activated cell sorting (FACS) analysis of NTERA2/D1, NCCIT, CHO-WT, and CHO-CR-1 cells. Cells were stained with PE-conjugated antibodies (normal mouse IgG and MAB2772P for CR-1). The CR-1^{High} population was defined so that the <2% cells were positive in the negative control staining. (D): Effect of serum on CR-1^{High} subpopulation. CR-1^{High} subpopulation was quantified by FACS. Mean $\pm$ SD is shown for four independent cultures. *, $p = .042$. (E): Effect of serum on pluripotency markers in NTERA2/D1 cells. Expression of the indicated genes was analyzed by quantitative reverse transcription-polymerase chain reaction. Mean $\pm$ SD is shown for three independent cultures. (F): Double immunofluorescence staining of CR-1 and pSmad2 protein in NTERA2/D1 cells. Nuclei were counterstained with DAPI. Arrowheads indicate the cells that highly express cell surface CR-1 protein and nuclear pSmad2. Scale bar = 20 μ m. (G): Western blot analysis of NTERA2/D1 cells. Proteins were extracted after cell sorting and analyzed for pSmad2 expression by Western blotting. Total Smad2 (tSmad2) is shown as a loading control. Abbreviations: CR-1, crypto-1; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast image; IgG, immunoglobulin G; PE, phycoerythrin; SSC, side scatter; WT, wild type.

Figure 2.

Characterization of CR-1^{High} and CR-1^{Low} subpopulations in NTERA2/D1 cells. (A): Postsort fluorescence-activated cell sorting (FACS) analysis of sorted CR-1^{High} and CR-1^{Low} subpopulations. (B): Representative phase-contrast images of CR-1^{High} and CR-1^{Low} cells cultured on plastic for 16 hours after cell sorting. (C): Different expression of undifferentiation or lineage markers between CR-1^{High} and CR-1^{Low} cells. Total RNA was purified from CR-1^{High} and CR-1^{Low} cells directly after cell sorting. Quantitative reverse transcription-polymerase chain reaction was performed for the indicated genes. Data were normalized with a housekeeping gene GAPDH. Relative values of CR-1^{High} versus CR-1^{Low} cells were measured from three independently sorted populations. (D): Coexpression of CR-1 and Oct4 in embryonal carcinoma (EC) cells in vitro. NTERA2/D1 cells were fixed and permeabilized, and then costained with CR-1 and Oct4. A representative FACS data are shown. (E): Coexpression of CR-1 and Oct4 in EC cells in vivo. Expression of CR-1 and Oct4 was analyzed by immunohistochemistry in xenograft teratocarcinoma tissue from NTERA2/D1 cells. (F): CR-1^{High} and CR-1^{Low} cells were cultured in suspension for 10 days. Tumor spheres with >50- μ m diameter were counted. Mean $\pm$ SD is shown for three independently sorted populations. *, $p < .05$; **, $p < .01$; compared with CR-1^{Low} cells. A representative phase-contrast image of tumor spheres from CR-1^{High} cells (right panel). Scale bar = 200 μ m. (G, H): In vivo xenograft assay. After cell sorting, 1,000 CR-1^{High} or CR-1^{Low} NTERA2/D1 cells were injected subcutaneously into nude mice. Mean $\pm$ standard error of tumor volume (G) and tumor-free survival (H) is shown ($n = 7$ for each population). *, $p = .035$. (I): Histological analysis of CR-1^{High} and CR-1^{Low}-derived tumors. Representative images for H&E (upper panel) and anti-CR-1 immunostaining (lower panel) are shown. (J): CR-1 expression in CR-1^{High} and CR-1^{Low}-derived tumors. CR-1 protein expression was quantified by Western blotting. $n = 4$ for CR-1^{High} and $n = 3$ for CR-1^{Low}. (K): Clonal analysis of CR-1^{High} and CR-1^{Low} populations. CR-1^{High} or CR-1^{Low} cells were seeded into 96-well plate at one cell per well directly after cell sorting. Wells containing only a single cell were marked and 3 weeks later, the established clones were analyzed for CR-1 expression by FACS analysis. Abbreviations: APC, allophycocyanin; CR-1, crypto-1; H&E, hematoxylin and eosin; PE, phycoerythrin; SSC-A, side scatter-area; SSC-H, side scatter-high.

Figure 3.

Regulation of CR-1 expression by Activin/Nodal signaling and by Oct4 and Nanog. **(A):** NTERA2/D1 cells were cultured in serum-free condition with or without Activin B (50 ng/ml), Nodal (250 ng/ml), or SB-431542 (10 μ M). After 3 days, cells were harvested, stained with anti-CR-1 antibody and fluorescence-activated cell sorting (FACS) analysis was performed as described above. Values represent mean $\pm$ SD of three independent experiments. *, $p < .05$; **, $p < .01$; compared with the control sample. **(B):** Western blot analysis for the efficiency of small interfering RNA (siRNA) knockdown of Oct4 and Nanog. **(C):** After 3 days of culture in the presence of serum, FACS analysis was performed for siRNA-transfected NTERA2/D1 cells. *, $p < .05$; compared with the control sample. **(D):** NTERA2/D1 cells were transfected with si-control, si-Oct4, or si-Nanog. After 24 hours, culture medium was replaced with serum-free medium with or without Activin B (50 ng/ml) or Nodal (250 ng/ml). Cells were cultured for 3 more days and FACS analysis was performed as described above. Values represent mean $\pm$ SD of three independent experiments. *, $p < .05$. **(E):** NTERA2/D1 cells were transfected with si-control, si-Oct4, or si-Nanog. After 24 hours, culture medium was replaced with serum-free medium with or without Activin B (50 ng/ml). Cells were cultured for 3 more days and total RNA was extracted. Quantitative reverse transcription-polymerase chain reaction was performed for the indicated genes. Data were normalized with GAPDH. Relative values compared with a si-control sample are shown. Abbreviations: CR-1, crypto-1.

Figure 4.

Direct regulation of CR-1 transcription by Oct4 and Nanog. **(A)**: Effect of exogenous expression of Oct4, Nanog, and Sox2 on CR-1 promoter activity in 293T cells. 293T cells were transfected with a full-length (4 kb) CR-1 promoter luciferase construct, renilla reporter (transfection control) and the indicated expression vectors (O/S, Oct4 + Sox2; O/S/N, Oct4 + Sox3 + Nanog). Values represent mean $\pm$ SD of fold induction over EV of relative light units from three independent experiments. *, $p < .05$; compared with EV. Expression of the transgenes was validated by Western blotting (inset). **(B)**: Effect of knockdown of Oct4 or Nanog on CR-1 promoter activity in NTERA2/D1 cells. Luciferase assay was performed with the indicated small interfering RNAs. Data were normalized with luciferase activity of pGL4 empty vector and mean $\pm$ SD from three independent experiments is shown. *, $p < .05$. **(C)**: Binding region of Oct4 and Nanog in CR-1 promoter. Chromatin immunoprecipitation (ChIP) was performed for Oct4 (top) and Nanog (bottom) in NTERA2/D1 cells using primer sets for various regions in the CR-1 promoter as shown in the diagram. Normalized values with the input DNA are shown. **(D–F)**: Binding of Oct4 and Nanog to the proximal region of the CR-1 promoter. ChIP was performed for control and RA-treated (5 days) NTERA2/D1 cells using the indicated antibodies. Isotype-matched normal IgGs were used for negative controls. The Nanog promoter was used as a known Oct4/Nanog binding region. GAPDH promoter was used as a control for Nanog/Oct4-independent, active promoter region. Abbreviations: CR-1, *cripto-1*; CR-1-FL, *cripto-1*-full length; EV, empty vector; IgG, immunoglobulin G; RA, retinoic acid.

Figure 5.

DNA methylation status of CR-1 promoter region. (A): Diagram of CpG sites within the 1.5 kb of the CR-1 promoter region. Regions analyzed by bisulfite sequencing are indicated (CpG#6–13 and CpG#37–42). (B): NTERA2/D1 cells were treated with 10- μ M RA for the indicated periods of time. CpG methylation was quantified by pyrosequencing after polymerase chain reaction amplification of bisulfite-treated genomic DNA. (C): CpG methylation was analyzed after fluorescence-activated cell sorting of NTERA2/D1. Abbreviations: CR-1, cripto-1; RA, retinoic acid; TSS, transcriptional start site.

Figure 6.

Effect of CR-1 knockdown on the expression of pluripotent stem cell-related genes. **(A)**: Validation of two small interfering RNAs (siRNAs) against CR-1 by Western blotting 48 hours after transfection. GFP expression vector and siRNA targeting firefly luciferase were used as controls. **(B)**: Forty-eight hours after siRNA transfection, RNAs were collected from NTERA2/D1 cells and analyzed for the indicated gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Values represent mean $\pm$ SD of three independent experiments. *, $p < .05$. **(C)**: NTERA2/D1 cells were treated with 10- μ M SB-431542 (SB) or 10- μ M RA for 48 hours. qRT-PCR was performed for the indicated genes. Values represent mean $\pm$ SD of three independent experiments. *, $p < .05$; **, $p < .01$; compared with control samples. Abbreviations: CR-1, cripto-1; GFP, green fluorescent protein; SB, SB-431542; RA, retinoic acid.

Figure 7.
A proposed model for regulation of CR-1 expression, pluripotency, and tumorigenicity in embryonal carcinoma cells. Abbreviation: CR-1, cripto-1.