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CRIPTO3, a presumed pseudogene, is expressed in cancer

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ABSTRACT

Cripto is a cell surface protein highly expressed in certain solid tumors, and overexpression of Cripto protein is oncogenic. Cripto-1 protein is encoded by CRIPTO1 gene. CRIPTO3, a presumed pseudogene, has an open reading frame with six amino acid differences from Cripto-1. We show that CRIPTO3 mRNA is the CRIPTO message expressed in many cancer samples. A CRIPTO3 SAGE tag was found in several cancer SAGE libraries, while the CRIPTO1 tag was found in ES cell libraries. *In vitro* experiments indicate both Cripto-1 and Cripto-3 proteins are functional in the Nodal-dependent signal pathway. Our data indicate that CRIPTO3 is an expressed gene, particularly in certain cancers, and suggest a potentially novel mechanism of oncogenesis through activation of a retrogene.

Cripto-1 is a cell surface associated EGF-CFC family protein that plays important roles in early development and cancer formation [1–4]. A loss of function mutation in CRIPTO1 is associated with holoprosencephaly in humans [5]. Mouse cripto1 is required for establishment of correct anterior-posterior axis in the embryo [6]. Cripto-1 is also a surface marker of embryonic stem cells [7].

Expression of Cripto protein in normal adult tissues is low, except in mammary gland, where Cripto may play a role in ductal epithelial cell differentiation [3]. However, Cripto protein is over-expressed in many human solid tumors [8–10], and Cripto over-expression is associated with metastasis of gastric cancer and poor prognosis of gastric and breast cancers [11,12]. It has been shown that mouse Cripto promotes squamous tumor growth [13], and *in vitro* study results indicate over-expression of Cripto leads to transformation of a normal mouse mammary epithelia cell line [9,14,15]. Several recent publications show that inhibition of Cripto either by monoclonal antibodies or anti-sense oligo-nucleotides inhibits cancer cell growth *in vitro* [8,16–18].

Cripto-1 protein activates its two sets of downstream target genes via two signaling pathways, the Nodal- and ALK4-dependent and -independent pathways. In the Nodal- and ALK4-dependent pathway, Cripto-1 protein binds to membrane bound ALK4 and Nodal, which lead to phosphorylation of transcription factor Smad2. The activated Smad2 and Smad4 factors then activate their downstream target genes [19,20]. In the Nodal- ALK4-independent pathway, Cripto protein binds to glypican-1, and activates the tyrosine kinase c-Src, which leads to phosphorylation and activation of downstream MAP kinase and AKT kinase [21]. Over-expression of Cripto-1 leads to uncontrolled growth of cells through several potential mechanisms, including activation of these signaling pathways and/or inhibition of TGF-beta signaling [4,13,22].

While it is clear that CRIPTO is expressed in many human cancer cell lines and tumors, and that CRIPTO over-expression is oncogenic, it is unclear how many genes encode Cripto protein. There are at least seven CRIPTO genes and pseudogenes in the human genome, named TDGF1 through TDGF7 (we use the names CRIPTO1 through CRIPTO7 in this paper). CRIPTO1 is widely believed the only structural gene for Cripto protein. Among the 6 pseudogenes, CRIPTO3 on the X chromosome has an intact open reading frame that encodes a predicted protein (Cripto-3) with 6 amino acid (AA) differences relative to published Cripto-1 protein reference sequence [23,24] (Fig. 1). CRIPTO3 is intronless, appears to be derived from an insertion of CRIPTO1 cDNA in the human genome during evolution, and is presumed not to be expressed. While early publications speculate on expression of CRIPTO3, only recently published work claims to have supportive data for its expression [25]. No direct experimental data has been published that establishes whether or not CRIPTO3 is expressed and translated. Consequently, all the Cripto expression has been assumed to be from the CRIPTO1 gene.

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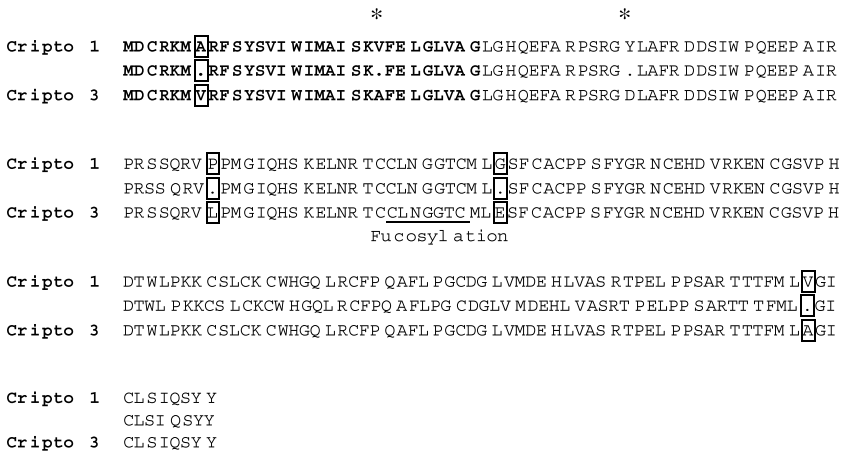

Fig. 1. Sequence alignment of proteins encoded by CRIPTO1 and CRIPTO3. The common sequence is in the middle. The positions with different amino acid residues between the two proteins are indicated by dots. The variable amino acid sites in Cripto-1 caused by SNPs are indicated with asterisks, and the sites with fixed amino acid differences between the two proteins are boxed. The signal peptide is in bold face. The potential fucosylation site is underlined.

During experiments to test anti-Cripto antibodies on different cancer cell lines, we observed apparent nucleotide sequence differences among CRIPTO mRNAs. Comparisons of genomic and cDNA sequences of CRIPTO from these cell lines revealed that the sequences of a large number of CRIPTO cDNA were identical to the CRIPTO3 sequence. This led us to test whether CRIPTO3 is indeed an expressed gene. Here we show that the CRIPTO3 gene is transcribed in a number of human tissues and cancer cell lines, particularly in cancer tissues, and Cripto-3 protein can be detected on cell membranes and can activate the Nodal-dependent signaling pathway.

Most tissue samples express either the CRIPTO1 or the CRIPTO3 gene. More importantly, CRIPTO3 is the only CRIPTO mRNA found in many primary cancer tissues. Analysis of public gene expression databases also supports the conclusion that CRIPTO3 is an expressed retrogene.

Materials and methods

Human genomic DNA and RNA samples. Human cancer genomic DNA and RNA was purchased from Biochain institute Inc. (Hayward, CA), or prepared from human tissues (Asterand, Detroit, MI) and cell cultures.

DNA was prepared with DNeasy kits (Qiagen, Valencia, CA). RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA) followed up with RNeasy kit (Qiagen, Valencia, CA). One microgram of each purchased RNA sample was digested by 1 U of DNase I (Invitrogen, Carlsbad, CA) for 15 min at room temperature prior to cDNA synthesis.

cDNA preparation and cloning. cDNA was synthesized with SMART cDNA synthesis kit (BD Clontech, San Jose, CA). cDNA from each sample was used as template to amplify full-length or fragment of CRIPTO1 and CRIPTO3 cDNA, with the primer pairs common to both genes: GGCTGAGTCTCCAGCTCAAGG (FL, forward) and GTATTTCTGGAAATAGGTCAATGTCG (FL, reverse); GGCTGAGTCTCCAGCTCAAGG (partial, forward) and TGTGATTTGGATCATGGCCA (partial, reverse). The PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA).

CRIPTO1 and CRIPTO3 specific PCRs. PCR conditions are as following: Initial denature at 95 °C for 1 min, then denature at 94 °C for 45 s, annealing at 64 °C (CRIPTO3 specific) or 66 °C (CRIPTO1 specific) for 45 s, and extension at 72 °C for 1 min, repeat for 35 cycles.

Forward primer for CRIPTO1: GCTACGACCTTCTGGGGAAAACG;
Reverse primer for CRIPTO1: CTGGTCATGAAATTTGCATG.

Forward primer for CRIPTO3: GCGTGTGCTGCCCATGGGA;
Reverse primer for CRIPTO 3: CGGGTCATGAAATTTGCATA.

PCR product size for CRIPTO1 fragment is 776 bp, for CRIPTO3 is 431 bp.

QPCR assay. The previous published protocol and gene specific oligos were used [25].

Fluorescence activated cell sorting (FACS) detection of Cripto proteins. Cells were incubated with 10 ug/ml of anti-Cripto antibody (B3F6), and stained with PE conjugated anti-IgG (Jackson Immuno Research, West Grove, PA). At least 10,000 cells were analyzed by flow cytometry, using FACS Calibur and FlowJo software.

293E cells were co-transfected using Fugene 6 (Roche, IN) with 2.5 μg of a GFP expression vector combined with 2.5 μg of either human CRIPTO1 or CRIPTO3 cloned into an expression vector whose control elements were derived from the pCEP4 EBV expression vector (Invitrogen, Carlsbad, CA). Cells were harvested 48 h later and analyzed by FACS for CRIPTO expression on GFP positive cells.

Cripto-Nodal signaling assay. Mouse F9 cells (cripto−/−, Nodal$^+$) at 2×10^5 cells/well in a 24 well plate were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) with 50 ng of $(n2)_7$-luciferase reporter construct, 100 ng forkhead activin signal transducer (FAST) transcription factor (downstream target of Cripto-Nodal pathway), and 100 ng full-length human CRIPTO1 or human CRIPTO3 expression plasmid, or empty expression vector. Forty-eight hours following transfection, the cells were lysed with LucLite (Perkin-Elmer, Wellesley, MA) and the luciferase activity was measured in a luminometer (Perkin-Elmer, Wellesley, MA).

DNA sequencing. DNA sequencing was done with ABI 3730 sequencer (Applied Biosystems, Foster City, CA).

Results

CRIPTO3 transcripts are found in cancer and normal tissues, as well as cancer cell lines

To investigate which CRIPTO gene is expressed in different human tumor tissues and human tumor cell lines, we PCR amplified CRIPTO cDNA from each sample using primers common to CRIPTO1 and CRIPTO3 cDNA, cloned the PCR products and sequenced multiple clones from each sample. Prior to PCR, the cDNA pools were tested for possible genomic DNA contamination by PCR with CRIPTO1 intron specific oligonucleotide primers. As shown in Fig. 2A, PCRs with intron specific primer pairs are negative from the cDNA

pools, and positive on genomic DNA controls. PCRs with GAPDH primers are positive for all these cDNA preparations (data not shown), indicating the negative PCR with CRIPTO1 intron specific primers is not due to lack of DNA, but rather lack of intron sequences (genomic DNA fragments). All of the isolates from cancer and matched normal control tissue (from the same patients) samples are derived from CRIPTO3, the presumed pseudogene (Supplementary Table 1).

To eliminate the possibility that the cloned cDNA fragments originated from RNA fragments that do not have coding potential, such as transcripts from truncated pseudogenes, we amplified full-length cDNAs using primers common to CRIPTO1 and CRIPTO3 from additional cancer tissue samples and did the same analysis. Essentially the same results were obtained, except CRIPTO1 cDNA isolates are found in a few normal breast and lung samples as well (Table 1). It is interesting to note that only CRIPTO3 cDNA clones were found in cancer tissues, while some normal breast and lung samples expressed both genes. Similar experiments were done on 10 cancer cell lines. Three of these cell lines express the CRIPTO3 gene only, and five lines predominantly express CRIPTO1, while two cell lines have very low level of CRIPTO expression (Supplementary Table 2).

To eliminate the possibility of PCR bias toward the transcript of one gene over the other, we capitalized on fixed nucleotide differences between CRIPTO1 and CRIPTO3 to perform transcript specific PCR on the same set of tissue cDNAs. As shown in Fig. 3, three normal breast and two normal lung samples express CRIPTO1, all cancer samples tested are positive for CRIPTO3 only or have negative RT-PCR result. This is consistent with the results of sequence analysis of cloned RT-PCR products from these samples.

Gene specific QPCR tests

We tested published CRIPTO1 and CRIPTO3 specific QPCR protocol [25] using CRIPTO1 or CRIPTO3 cDNA, as well as a CRIPTO1 and CRIPTO3 cDNA mixture, as templates. In our hands, the CRIPTO1 oligos are specific to CRIPTO1 cDNA, while the reported CRIPTO3 specific oligos cannot distinguish CRIPTO1 and CRIPTO3 cDNA template (Supplementary Fig. 1). Additional oligos were designed and tested with multiple QPCR conditions, none is found specific to CRIPTO3.

Gene expression data in SAGE and Affymetrix profiling databases suggest CRIPTO3 gene is expressed in some solid tumors

Searching public SAGE (Serial Analysis of Gene Expression) databases generated independent evidence of CRIPTO3 expression. With a CRIPTO1 SAGE tag sequence (TAATTCTACCAAGGTCT) and a CRIPTO3 SAGE tag sequence (CTCTTCAGAA), two non-overlapping set of libraries were found positive for either the CRIPTO1 or the CRIPTO3 tag (http://www.ncbi.nlm.nih.gov/projects/SAGE/index.cgi?cmd=tagsearch). Five SAGE libraries were positive for CRIPTO3, four of which are from cancer samples and one from human bone marrow CD34+ cells. Ten libraries were positive for CRIPTO1, nine of which

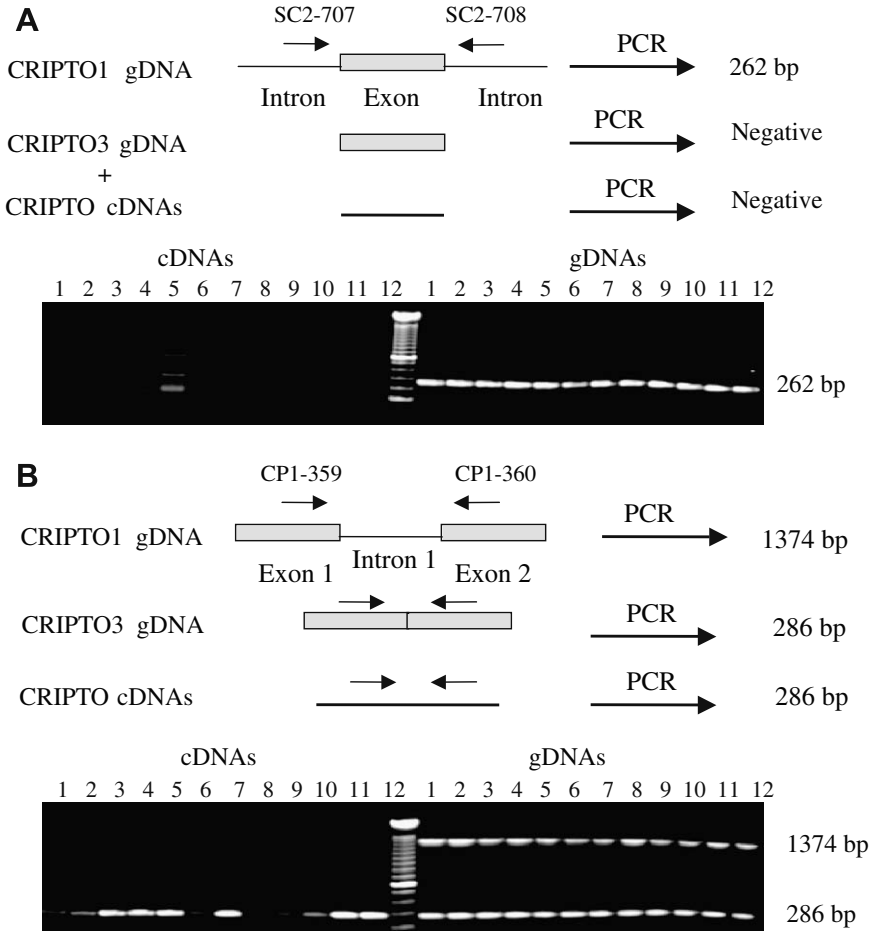

Fig. 2. PCR amplification of CRIPTO cDNA fragment. Positions of primers relative to CRIPTO genes and transcripts, as well as the expected outcome from PCRs with cDNA and genomic DNAs are diagramed. (A) cDNA purity test. Lane 1–12: cDNA from tumors. Lane 1–4: breast tumors; lane 5–8: colon tumors; lane 9–12: lung tumors, lane 13: 100 bp DNA marker, lane 14–25: genomic DNA from same set of tissue samples as in lane 1–12. (B) Inter-exon PCRs. The 1374 bp fragment is from CRIPTO1, the 286 bp fragment amplified from genomic DNA is from CRIPTO3 gene. The 286 bp fragment amplified from cDNAs is from CRIPTO1 and/or CRIPTO3 cDNA.

Table 1
Summary of full-length CRIPTO cDNA TA clones from cancer tissues

Tissues	Total number of samples	CRIPTO positive samples	Number of CRIPTO clones	Number of CRIPTO1 clones	Number of CRIPTO3 clones
Colon cancer	12	3	35	0	35
Normal colon	6	3	31	0	31
Breast cancer	12	8	111	0	111
Normal breast	6	4	75	70	5
Lung cancer	12	5	55	0	55
Normal lung	6	5	73	20	53

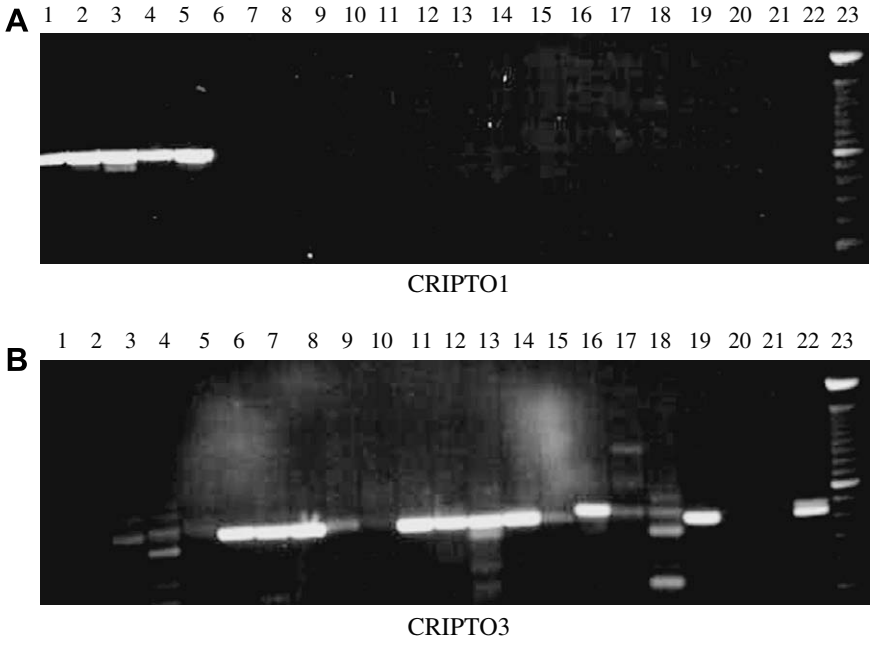

Fig. 3. Transcript specific PCRs. (A) PCR with CRIPTO1 transcript specific primer pairs; B. PCR with CRIPTO3 specific primer pairs. Lane 1–3, normal breast samples; lane 4–5, normal lung samples; lane 6–12, breast cancers; lane 13, normal colon; lane 14–15, colon cancers; lane 16–18, normal lungs; lane 19, normal matched lung; lane 20–21, lung cancers; lane 22, gDNA control; lane 23, 100 bp DNA markers.

are from embryonic stem cells and one from fetal brain. This is consistent with known involvement of CRIPTO1 in embryonic development and expression of Cripto-1 in stem cells, and consistent with our finding of CRIPTO3 expression in cancer.

Further supporting evidence comes from mining cancer gene expression data. Probe set 40386_r_at (16 probes) on the human U95Aver2 Affymetrix chip is annotated as CRIPTO1 specific, however, only one of the 16 probes is actually specific for CRIPTO1, all the other 15 probes are common to CRIPTO1 and CRIPTO3 gene. CRIPTO expression is found in 28 of 42 human malignant colon tumor samples with this probe set (samples hybridized by GeneLogic Inc.). Interestingly, the signal from the true CRIPTO1 specific probe is almost always "absent." ($p < 0.01$). The lack of signal from this probe could be due to (1) this particular oligonucleotide cannot hybridize well under the hybridization conditions used; (2) most CRIPTO transcripts in these cancer samples are from CRIPTO3. Currently we do not have a microarray assay to distinguish between these possibilities, nevertheless, this result is consistent with the hypothesis that CRIPTO3 is the gene expressed in most of the CRIPTO expression positive cancer tissues.

Cripto-3 protein is expressed in certain cells and is functional in Nodal signal pathway

To address the question whether CRIPTO3 mRNA is actually translated in cells and Cripto-3 protein transported to cell surface, we performed FACS experiments on CRIPTO1 and CRIPTO3 expressing cell lines with an antibody that recognizes a common epitope of both proteins. Results of FACS staining of BT474 cells, in which only CRIPTO3 transcript was detected by RT-PCR, are very similar to that of NCCIT cells, which expresses CRIPTO1 only (Fig. 4A and B). These data indicate that the endogenous CRIPTO3 gene is transcribed, and that protein is produced and translocated to the cell surface of BT474 cells. We also performed FACS on CRIPTO1 or CRIPTO3 transfected 293e cell lines, a cell line that does not express endogenous CRIPTO genes, and obtained similar results (Fig. 4C and D).

We tested human Cripto-1 and Cripto-3 proteins for their ability to signal through Nodal in a FAST transcription factor dependent $(n2)_7$-luciferase reporter assay. Activity was assessed in a mouse F9 derived embryonic carcinoma cell line that has the cripto gene disrupted (F9 cripto−/−). This cell line has wild type Nodal and all other genes in the Nodal-dependent Cripto signal pathway. There is a 4- to 6-fold increase of luciferase activity in CRIPTO3 and CRIPTO1 transfected cells when compared with negative (no CRIPTO) control (Fig. 4E), indicating human Cripto-1 and Cripto-3 are both capable of signaling through the Nodal-dependent pathway.

Discussion

In this report, we show evidence that the presumed pseudogene CRIPTO3 is a functional retrogene. CRIPTO3 mRNA is expressed in a variety of human tissues and cell lines. Furthermore, in cell lines

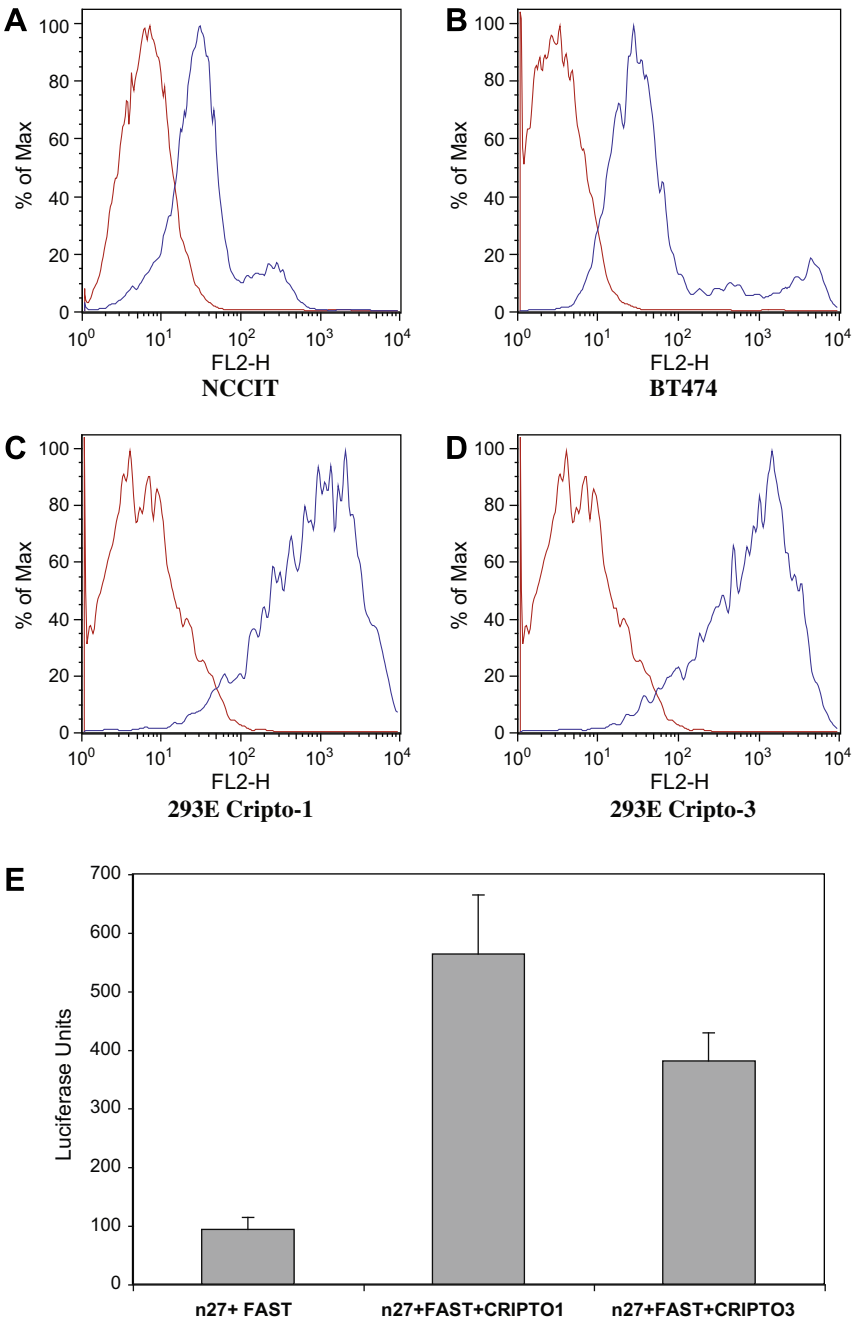

Fig. 4. Cripto-3 gene is expressed and functional in Nodal signal pathway. (A–D) FACS of Cripto-1 or Cripto-3 positive cell lines with an antibody (B3F6) against Cripto proteins. (A) NCCIT (Cripto-1 positive), (B) BT474 (Cripto-3 positive), (C). 293e cell transfected with CRIPTO1, and (D) 293e cell transfected with CRIPTO3. Red lines are the negative controls with PE labeled anti-IgG, and (E) Cripto-Nodal signaling assays. Mouse F9 Cripto−/− cells were co-transfected with plasmids expressing $(n2)_7$-luciferase and FAST (column 1) and human CRIPTO1 (column 2) or human CRIPTO3 (column 3).

expressing only endogenous or transfected CRIPTO3 mRNA, Cripto-3 protein is made and translocated to the cell surface. Cripto-3 protein is functionally equivalent to Cripto-1 in Nodal signaling assays. Thus there are two functional genes in the human CRIPTO gene family, CRIPTO1, which encodes the Cripto-1 protein, and CRIPTO3, which encodes the Cripto-3 protein.

CRIPTO genes and proteins are of general and medical significance for several reasons (1) Mutations in CRIPTO1 are associated with developmental defects [5,6]; (2) Cripto-1 is a cell surface marker for human embryonic stem cells [26]; (3) Engineered overexpression of Cripto-1 is oncogenic [14,27,28]); (4) Cripto protein is expressed on the surface of some tumors [3,9,10], and is a target for therapeutic antibodies [8,18]. It is noteworthy that all of the CRIPTO-expressing cancer tissues assayed in this study express CRIPTO3 mRNA exclusively or at levels much higher than that of CRIPTO1.

It is clear that CRIPTO1 plays a key role in stem cell biology and during early embryonic development [5–7], and CRIPTO1 expression has long been thought to associate with cancer. It is therefore intriguing that most CRIPTO3 expression observed in our study is in cancer tissues. The amino acid sequences of Cripto-1 and Cripto-3 are very similar, and both proteins are capable of signaling through the Nodal pathway. Thus it is possible that Cripto-3 protein has tumor promoting properties similar to Cripto-1.

Expression of CRIPTO3 may have implications for selection of patients for anti-Cripto therapy. Fixed sequence differences be-

tween Cripto-1 and Cripto-3 proteins make it possible for development of diagnostic tests to know which gene is expressed in a given tumor. Thus the spectrum of diagnostic options may be expanded to include RT-PCR as well as IHC. Further genomic analysis may also shed light on how the CRIPTO3 gene is de-regulated or up-regulated in cancers, and which roles it plays in tumorigenesis, thus giving us more information about how to target Cripto protein or its interacting proteins for cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.113.

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