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Cripto: roles in mammary cell growth, survival, differentiation and transformation

Christina C. Niemeyer[1,4], M. Graziella Persico[2] and Eileen D. Adamson[1,3]

[1] The Burnham Institute, La Jolla Cancer Research Center, 10901 North Torrey Pines Road, La Jolla, California 92037, USA
[2] International Institute Genetics and Biophysics, Naples, Italy
[3] corresponding author: Eileen Adamson. tel: (619) 455-6480; fax: (619) 646-3195; e-mail: eadamson@ljcrf.edu
[4] Present address: Structural Bioinformatics Inc., 10929 Technology Place, San Diego. 92127, USA

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Abstract

Cripto-1 (Cr-1) protein, encoded by the teratocarcinoma-derived growth factor gene (*TDGF-1*), is highly correlated with transformation in breast cancer. Eighty-two percent of breast carcinomas express Cr-1 whereas it is undetected in normal human breast tissue. We confirmed and extended findings that Cr-1 protein is expressed during the pregnancy and lactating stages of normal murine mammary glands but is barely detectable in glands from virgin animals and is undetectable in involuted glands. Cr-1 was found to be expressed in CID 9 cells, a line of mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Exogenous mouse Cr-1 expression from a retroviral vector caused CID 9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID 9 cells over-expressing Cr-1 did not differentiate efficiently. Infection of CID 9 cells with a Cr-1 antisense vector caused these cells to change in morphology, to grow slowly, to undergo apoptosis at a higher rate and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cr-1 is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of differentiation. In transplantation studies, Cr-1 over-expression stimulated the growth and survival of mammary cells, but did not stimulate tumorigenesis *in vivo*.

Keywords: retroviruses; overexpression; antisense; milk proteins; apoptosis; anchorage independent growth; tumorigenicity

Abbreviations: Cr-1 or CR-1, Cripto; DMEM, Dulbeccos minimum essential medium; EGF, epidermal growth factor; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbant assay; FBS, fetal bovine serum; IRES, internal ribosome entry site, PBS, phosphate-buffered saline; TDGF-1, teratocarcinoma derived growth factor-1 gene

Introduction

Cripto (Cr-1) was first assigned to the Epidermal Growth Factor (EGF)-like family of ligands that includes transforming growth factor-alpha (TGFα), and amphiregulin (AR). This family of proteins contain 'EGF-like domains' with a highly conserved structure of three disulfide loops, and in Cr-1, the six conserved cysteines that make up the EGF motif lacks the A-loop and the B-loop is truncated. As a result, Cr-1 does not bind to the EGFR or members of the ErbB family of receptors. At the carboxyterminus of Cr-1, an additional six-cysteine motif is present that is conserved in an analogous protein recently isolated from *Xenopus laevis* (Kinoshita *et al*, 1995). A related mouse protein, Cryptic, was recently cloned and this group of genes forms a new family named CFC (Shen *et al*, 1997). Mouse Cr-1 protein consists of 171 amino acids and unlike the human CR-1 (188 amino acids) has a signal sequence and is secreted (Brandt *et al*, 1994) (Dono *et al*, 1993) (Normanno *et al*, 1994) (Ciccodicola *et al*, 1989).

CR-1 was cloned as a full length isolate from a cDNA library derived from the human teratocarcinoma cell line NTERA2 clone D1. The TDGF-1 (CR-1) gene and an intronless sequence CR-3 were isolated and mapped on human chromosomes 3 and X, respectively (Dono *et al*, 1991; Saccone *et al*, 1995). Mouse *Tdgf1* (encoding Cr-1), and two intronless pseudogenes, *Tdgf2* and *Tdgf3*, have been isolated and characterized (Dono *et al*, 1993; Liguori *et al*, 1996). Mouse Cr-1 has 93% similarity to its human counterpart in the EGF-like domain, which is the most conserved. The molecular mass of Cripto protein varies according to the species and cell type: in human GEO colon and NTERA2/D1 embryonal carcinoma cells a polypeptide of 36 kDa predominates and it can be differently processed by glycosylation or modified in other ways. In mouse F9 cells only the secreted protein has been analyzed and a single species at 24 kDa was noted (Brandt *et al*, 1994).

CR-1 protein is highly correlated with tumorigenicity. Of 68 biopsies on breast carcinomas, 82% expressed Cripto. Cripto expression has not been detected in normal human breast tissue or cell lines (Qi *et al*, 1994). Growth of two human breast carcinoma cell lines and the nontransformed human epithelial cell line 184A1N4 was stimulated by the addition of synthetic refolded human CR-1 peptides containing the EGF-like domain (Brandt *et al*, 1994). All the human mammary tumor cell lines examined were found to express CR-1 using RT–PCR, Northern blot analysis and immunocytochemistry (Normanno *et al*, 1994). The human CR-1 cDNA has been overexpressed in mouse NIH3T3 cells and was shown to transform these cells such that they grew in soft agar in contrast to control cells

(Ciccodicola *et al*, 1989). The same construct transformed an immortal mouse mammary cell line, NOG-8 (Ciccodicola *et al*, 1989). Mammary tumors formed in transgenic mice overexpressing oncogenes TGFα, neu, int-3, polyomavirus middle T antigen or simian virus 40 large T antigen, all express Cripto-1 (Kenney *et al*, 1995). In addition, Cr-1 expression was observed in pregnant and lactating mouse mammary glands (Kenney *et al*, 1995). The latter observation suggested that Cripto played a role in differentiation as well as proliferation.

In the present study, CID 9 cells were used to examine the expression of Cripto-1 and to determine the effects of over- and under-expression of this growth factor during growth *versus* differentiation. CID 9 cells are a subpopulation (Schmidhauser *et al*, 1990) of the COMMA-1D mammary epithelial cell line which was established from normal 14.5 day pregnant Balb/c mouse mammary gland tissue. They retain important characteristics of normal morphogenesis and functional differentiation *in vitro* (Danielson *et al*, 1984). Normal CID 9 cells differentiate into alveolar-like structures (mammospheres) that express β-casein when grown on a laminin-rich extracellular matrix in the absence of fetal bovine serum (FBS) and in the presence of lactogenic hormones (Schmidhauser *et al*, 1990).

We show here that Cr-1 is differentially expressed during mammary gland development and is expressed in a hormone dependent fashion in the normal mammary epithelial cell line. Inhibition of Cr-1 expression caused a change in cell morphology, decreased cell growth, increased programmed cell death and reduced anchorage independent growth. Mouse Cr-1 overexpression stimulated anchorage dependent and independent cell growth and decreased the differentiation potential of the mammary cells. However, no increases in the tumorigenicity of Cr-1 over-expressing mammary cells were noted after transplantation into syngeneic hosts.

Results

Cripto is a pregnancy and lactation stage specific protein

Cripto protein, Mr 24 and 26 kDa, is strongly expressed in the second phase of mammary gland development, pregnancy (Figure 1, lane 2 shows Cr-1 in 14.5 day pregnant glands). This implies that its expression is driven by pregnancy-associated hormones, since involution of mammary gland tissue after pregnancy is associated with loss of Cripto expression (Figure 1, lane 4). In contrast, the virgin mouse mammary gland expresses extremely low levels (Figure 1, lane 1) in partial agreement with Kenney *et al*. (1995).

Cr-1 is also expressed in CID 9 cells, derived from 14.5 day pregnant mammary glands, as a 24 kDa protein (Figure 1, lane 7). When the cells were cultured on basement membrane substrates, they differentiated, observed as mammosphere and expression of casein, and they also expressed higher levels of Cr-1 protein. The Cr-1 protein was also modified to Mr 26 and 28 kDa forms (Figure 1, lane 6). The CID 9 mammosphere structures appeared to be functionally similar to the *in vivo* pregnant-lactating mammary gland with stage-specific Cripto gene expression. This observation prompted us to test the role of Cr-1 by experimental manipulation of C-1 expression in CID 9 cells.

Expression of exogenous Cripto

In order to affect the expression of Cripto in mammary cells, a retroviral vector containing either sense or antisense Cripto was constructed. The polycistronic retroviral vector pGCEN (Figure 2) contains the encephalomyocarditis virus internal ribosome entry site (IRES) which allows efficient expression of multiple genes from a single proviral genome. Transcriptional controls and RNA processing steps that differentially affect expression of the exogenous genes can be avoided (Ghattas *et al*, 1991). Cripto cDNA sequences (both sense and antisense) were inserted as described in the Materials and Methods section. Cripto cDNA and the selectable marker, neo^r are both expressed from a single promoter using the EMCV IRES insert. The pGCEN vector LTR is a promoter known to function in the mammary gland (Bradbury *et al*, 1991). CID 9 cells were infected with the retroviruses. Because the CID 9 cells are a heterogeneous cell

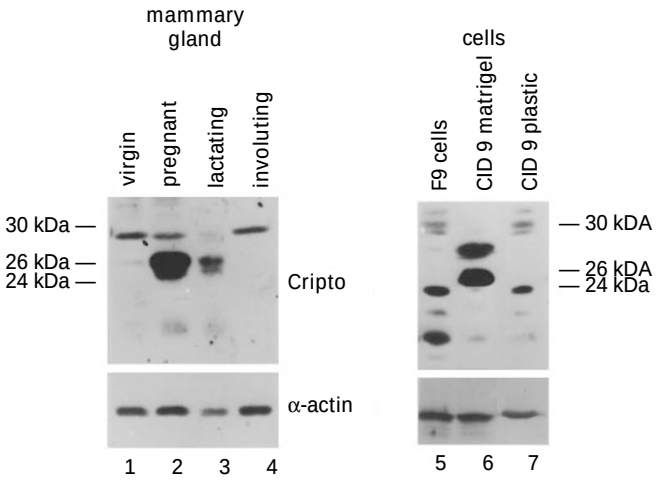

Figure 1 Immunoblots of normal mammary gland tissue and cells to show Cripto protein expression. Cripto is highly expressed as 24 and 26 kDa proteins in 14.5 day pregnant gland (lane 2) and at a lower level at the lactating stage (lane 3) of mammary gland development. It is expressed at very low levels in virgin glands (lane 1) and is not seen in involuting glands (lane 4). Cripto is expressed as a 24 kDa protein in undifferentiated F9 cells (lane 5) and as both 26 and 28 kDa proteins in CID 9 cells grown on an extracellular matrix (Matrigel) for 7 days in the presence of lactogenic hormones (lane 6), compared to CID 9 cells grown in maintenance media on plastic (lane 7). Other bands in the figure are non-specific bands

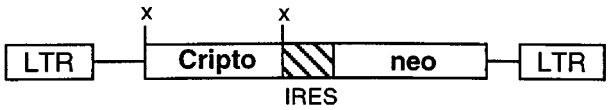

Figure 2 pGCEN retroviral expression vector used to over and under express Cripto in mammary cells. The Cripto cDNA was inserted in both orientations at the *Xho*I site (X). IRES, encephalomyocarditis virus internal ribosome entry site; LTR, Moloney murine leukemia virus Long Terminal Repeat sequences; neo, bacterial neomycin resistance gene

population, more than 250 clones were selected in G418 and then pooled.

The level of Cripto protein expression in the infected cells was measured using Western blot analysis. Two different populations of CID 9 cells containing Cripto in the sense orientation were analyzed (Figure 3) and showed that Cripto was overexpressed at levels greater than twofold higher than control vector populations (Figure 3). In the antisense populations only approximately one-quarter of the amount of Cripto was expressed compared to the controls (Figure 3). Thus, the retroviral vector constructs effectively modulated Cr-1 expression in CID 9 cells. Both cytokeratin positive and vimentin positive cells were observed, using indirect immunofluorescent staining, in all the populations. The proportion of keratin-positive cells was similar in all three populations, amounting to $55 \pm 10\%$. Thus, there was no selection of one population and all the cell lines contained the two distinct cell types described by Desprez (Desprez *et al*, 1993).

Morphology

Monolayer cultures of CID 9 cells commonly show two cellular morphologies, a spindle-shaped and a typical epithelial cobblestone pattern (Figure 4A). No differences were observed in the mixed morphology of cells after infection with the empty vector compared to uninfected CID 9 cells (Figure 4A and B). The cells infected with the sense Cripto cDNA also exhibited the same cellular morphology as the CID 9 cells or the pGCEN control CID 9 cells at both high and low cell densities. No significant morphological differences were observed during continuous propagation of these various pools. At low density, cells grew as islands of cells. In cultures remaining at confluence for 2 days, the presence of domelike structures was apparent. Cr-1 overexpressing cells formed more frequent and larger domelike structures (Figure 4C) compared to wt cells. Cells expressing antisense Cripto became morphologically distinct; the individual cells were flatter and spread to a larger area at both high and low density. They showed the more typical cobblestone morphology of epithelial cells (Figure 4D) and at confluence, they did not form domes. However, like the parental cells, they still contained at least two cell types that expressed keratin or vimentin intermediate filaments (Desprez *et al*, 1993), and did not represent a specifically selected population.

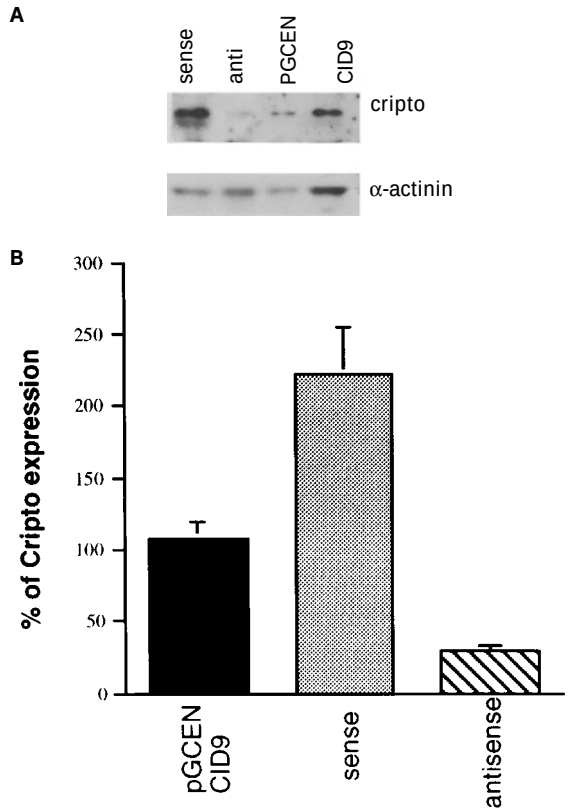

Figure 3 Cripto expression levels in infected CID 9 cells. (**A**) Overexpression of Cripto in CID 9 cells infected with the pGCEN-sense Cripto vector (sense) and underexpression in cells infected with the pGCEN-antisense Cripto vector (anti). α-Actinin was used to show relative protein levels in each lane. (**B**) Graph of the percentage of Cripto expression $\pm$ S.D. in two different populations of infected cells compared to control pGCEN vector infected CID 9 cells (pGCEN) or uninfected CID 9 cells (CID 9) which was defined as 100%

Cell proliferation and apoptosis

To determine if over- and under-expression of Cr-1 had an effect on mammary cell growth, several types of growth assays were performed. Cell growth rates were determined using maintenance culture medium. In this media, the CID 9 cells overexpressing Cripto (sense), underexpressing Cripto (antisense), and the control cells containing the empty vector (pGCEN) all grew at approximately the same rate for the first 24 h. Then the cells overexpressing Cripto grew faster so that at 48 and 72 h there were a greater number of cells compared to the control. The cells with reduced Cripto appeared to reach confluence by 24 h and the level of cells subsequently decreased after 48 h suggesting that they were starting to die (Figure 5A).

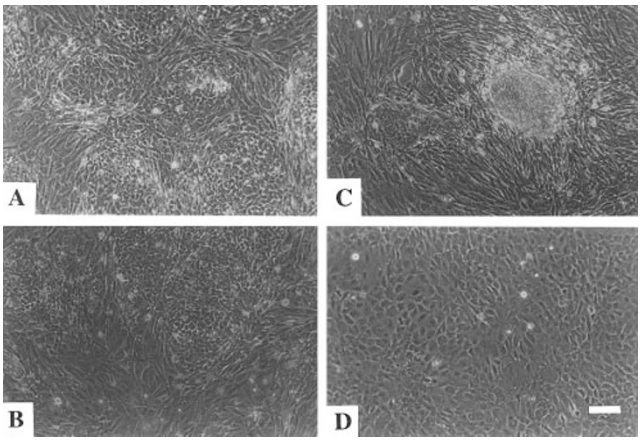

Figure 4 Morphological effect of Cripto over- and under-expression on CID 9 cells. Phase contrast micrographs compare the general appearance of the (**A**) normal CID 9 cells with (**B**) cells infected with control pGCEN vector, and (**C**) cells infected with the Cripto expression vector, and (**D**) cells infected with the antisense Cripto expression vector. All the cells were seeded at the same density and allowed to grow the same length of time. For all panels the bar indicates 100 μm

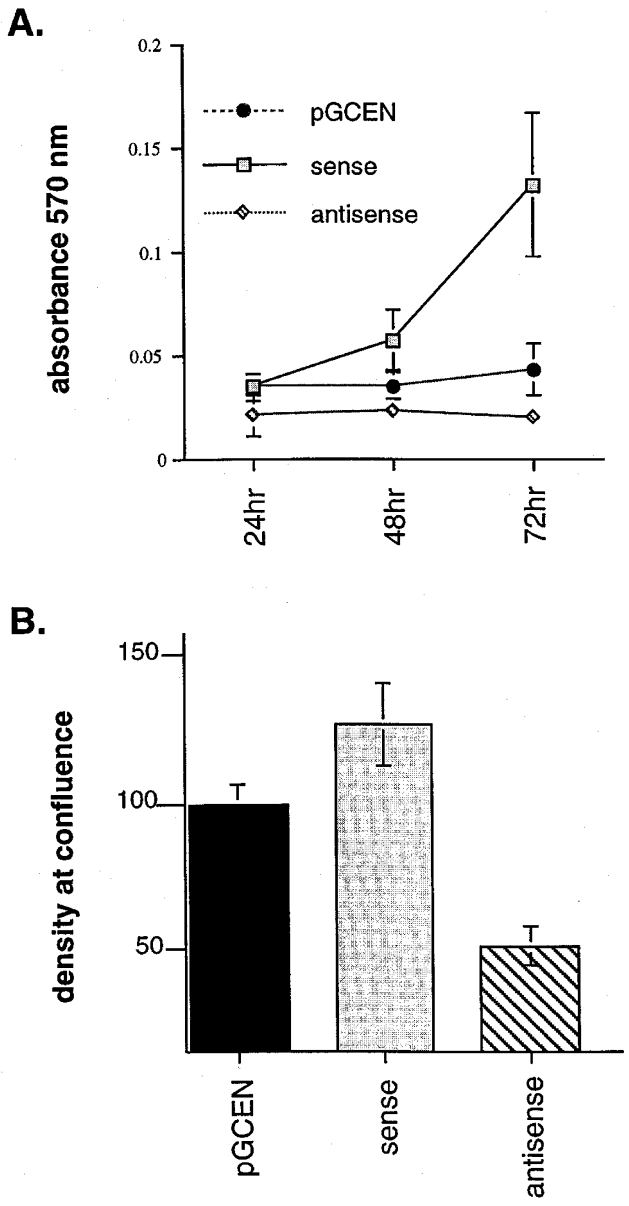

Figure 5 Cell growth assays. Growth rates of CID 9 cells containing the empty vector (pGCEN), the Cr-1 expression vector (sense), and the antisense Cr-1 expression vector (antisense). (**A**) The absorbance at 570 nm is directly proportional to the number of cells/well. (**B**) Plateau growth density of cells expressing various levels of Cr-1. Values are expressed as a percentage of the CID 9 cells containing the control pGCEN vector. Bars, S.D.

To determine cell densities at confluence, cells were grown in 2% fetal bovine serum (FBS), allowed to grow for 5 days and remain at confluence for 2 days and then counted (Figure 5B). The Cr-1 overexpressing cells grew to a greater density (3.2×10^5 cells/cm^2) than the control cells (2.4×10^5 cells/cm^2). For CID 9 cells expressing decreased levels of Cr-1, the density at confluence remained at 1.2×10^5 cells/cm^2. Thus the cells containing the antisense vector showed contact inhibition at a statistically significant ($P < 0.05$) lower cell density compared to parental and control cells.

To determine if Cr-1 lowered the requirement of CID 9 cells for growth factors, cell proliferation assays were also performed by plating the cells in media containing 2% FBS to allow attachment and then growing them in serum-free conditions. Uninfected CID 9 cells and cells containing the sense or empty vector grew at approximately the same rate and all the rates were lower than in 2% or 5% FBS conditions. The CID 9 cells containing the retrovirus with Cr-1 in the antisense orientation showed no growth in serum-free media and after several days no cells survived (data not shown).

This observation suggested that the loss of Cr-1 expression might affect survival of cells even in serum-containing cultures and this was tested next. Apoptosis levels were assessed by nuclear morphology and by TUNEL assays (Figure 6a). Numbers of apoptotic nuclei (Figure 6b) in log phase CID 9 cells were $0.60\% \pm 0.20$ of the population in control pGCEN cells; this rate decreased to $0.28\% \pm 0.16$ ($P < 0.05$) in Cripto overexpressing cells and increased to $1.53\% \pm 0.66$ ($P = 0.035$) in antisense cells. More frequent apoptotic nuclei were observed in antisense-expressing cells (Figure 6a, E, F) than in either of the other two cell populations (Figure 6a, A, B, C, D). The expression of Cripto therefore endows a growth advantage as well as better survival in cultured mammary cells.

Colony formation and anchorage independent growth

The Cripto overexpressing sense CID 9 cells have a greater tendency to build up multilayers of cells in monolayer cultures compared to control pGCEN infected or uninfected CID 9 cells (Figure 7A and B). The normal cells formed colony-like domes but CID 9 cells underexpressing Cr-1, on the other hand, showed very little colony formation compared to control cells and ceased to proliferate after reaching confluence (Figure 7C). The three CID 9 populations showed differential abilities to grow in soft agar (Figure 7). Control cells formed 15 ± 3 colonies per dish or 0.04% of the plated cells formed colonies (Figure 7D). Whereas the Cripto overexpressing cells formed greater than 79 ± 6 colonies or 0.2% of plated cells formed colonies (Figure 7E). The antisense cells did not grow in soft agar and were therefore anchorage dependent for growth (Figure 7F).

Differentiation

CID 9 cells differentiate efficiently on an extracellular matrix in the presence of lactogenic hormones. Our three populations, containing either the sense, antisense, or empty pGCEN vector, were allowed to differentiate on Matrigel^{TM}, where morphological differentiation can be observed by the ability to form polarized epithelial structures termed mammospheres. The empty pGCEN infected CID 9 cells appeared the same as the parental CID 9 cells in that they formed frequent mammosphere structures (Figure 8A). The CID 9 cells overexpressing Cr-1 grew faster than the control cells (data not shown) and there were overlapping cell layers, however, no mammosphere structures formed (Figure 8B). This suggests that overexpression of Cr-1 in cells overrides the

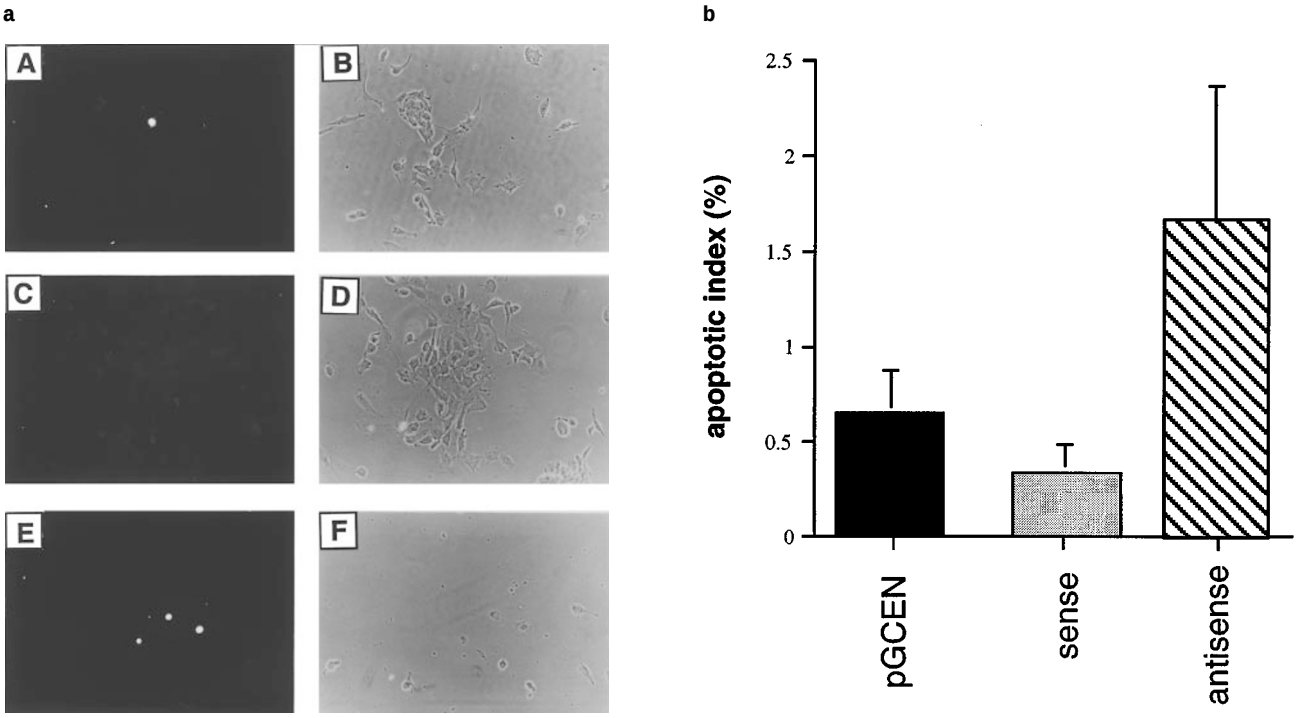

Figure 6 The effect of Cr-1 on CID9 cell survival. (**a**) *In situ* detection of apoptotic cells with the TUNEL method (left panels, A, C, E) and phase contrast microscopy (right panels, B, D, F) of CID 9 cells expressing various levels of Cr-1. All cells were plated at the same density and grown under the same conditions. A, B, control cells containing the empty pGCEN vector; C, D, CID9 cells overexpressing Cr-1 (sense); E, F, cells underexpressing Cr-1 (antisense). One representative field of each population is shown. (**b**) Apoptotic levels of log phase CID 9 populations (see Materials and Methods for details). Bars, S.D.

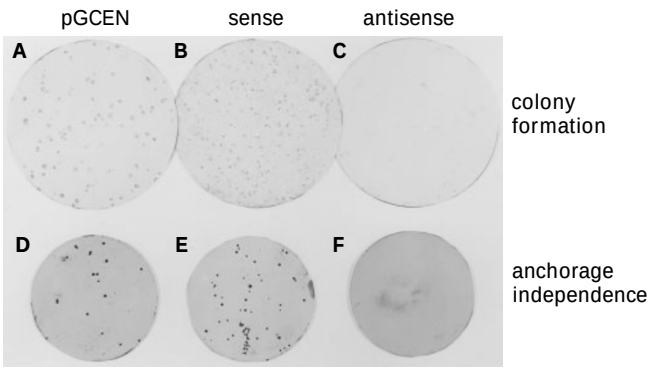

Figure 7 Colony formation in monolayer culture and anchorage independence (soft agar) assays of various populations. The CID 9 cells containing the empty vector (pGCEN) show some colonies formed both on plastic (**A**) and in soft agar (**D**). CID 9 cells overexpressing Cr-1 (sense) show a threefold greater number of colonies both on plastic (**B**) and in soft agar (**E**). Whereas, CID 9 cells underexpressing Cr-1 (antisense) show very little or no colony formation on plastic (**C**) or in soft agar (**F**)

signals leading to differentiation that normally appear. Interestingly, antisense expressing cells also did not form any mammospheres but tended to aggregate into aster-like structures (Figure 8C).

Biochemical indications for differentiation in mammary cells are the syntheses of milk proteins including β-casein. The CID 9 cells were grown in the presence of the lactogenic hormones, insulin, prolactin, and hydrocortisone either on an extracellular matrix or on plastic, and β-casein was detected by immunoblotting with an antibody to mouse milk proteins. Equal loading of gels was verified by immunoblotting with an antibody to α-actinin (Figure 9, lower panel) and the levels of Cr-1 produced in the cells collected after the experiment was confirmed by immunoblotting with anti-Cr-1 (Figure 9, upper panel). The CID 9 cells overexpressing Cr-1 showed only about half the amount of β-casein expression compared to the control CID 9 population (Figure 9, lanes 2, 3, 11, and 12). This agrees with our morphological observations that Cr-1 overexpressing cells are predominantly proliferating compared to the control or normal CID 9 cells. Unexpectedly, the antisense containing CID 9 cells showed equal or greater expression of β-casein compared to the control suggesting (a) that Cr-1 is not involved in β-casein expression and (b) that mammospheres are not important for differentiated expression. The addition of matrix is not necessary for casein expression (Figure 9, lanes 10–12), probably because CID9 cells synthesized their own matrix during this time. All populations of CID 9 cells (sense, antisense or empty vector) if grown in maintenance media without hydrocortisone and prolactin, either to confluence or not, failed to express β-casein (Figure 9, lanes 4–9).

A milk protein of approximately 22 kDa whose regulation was distinct from the control of β-casein expression (Marte *et al*, 1995a) was produced by the CID 9 cells. Unlike β-casein expression, this protein was expressed in the cells whether lactogenic hormones were present or not. This

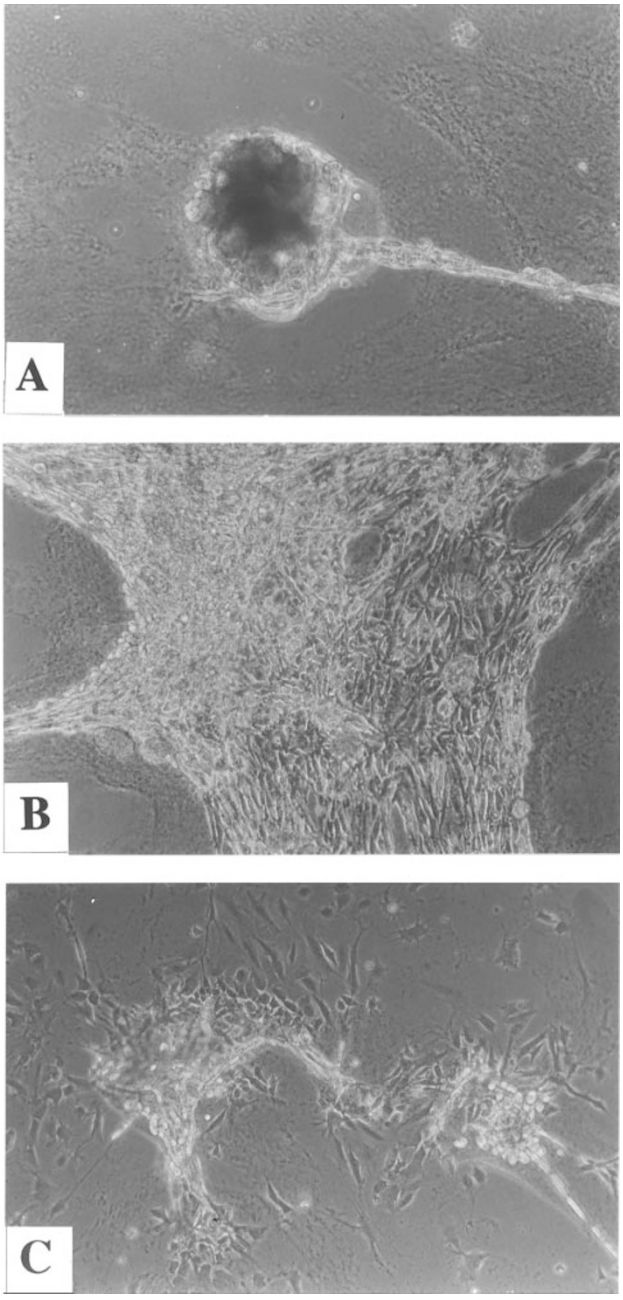

Figure 8 Morphological appearance of CID 9 cell populations grown on extracellular matrix (Matrigel) in the presence of lactogenic hormones. Phase contrast micrographs showing (**A**) normal differentiated structures (mammospheres) formed in control population of CID 9 cells containing the empty pGCEN vector, (**B**) overgrowth of CID 9 cells overexpressing Cr-1, no mammosphere structures were observed and (**C**) aster-like structures formed when decreased levels of Cripto were expressed. All the cells were seeded at the same density and allowed to grow for 6 days

protein was expressed at approximately the same level in all cell populations except Cr-1 overexpressing CID 9, which expressed only 15% of the level of expression of the 22 kDa protein observed in the other populations (Figure 9, lane 2). This property further distinguished the Cr-1 overexpressing cells.

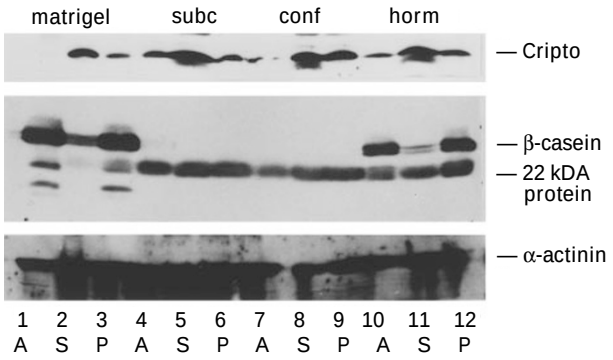

Figure 9 Immunoblots to show proteins produced by populations of CID 9 cells grown under various conditions. CID 9 cells underexpressing Cr-1 (**A**), overexpressing Cr-1 (S) or expressing normal levels of Cr-1 and containing the empty vector (P) were analyzed. Growth conditions included growth on Matrigel in the presence of lactogenic hormones for 6 days (Matrigel, lanes 1–3); growth on plastic in normal maintenance media until they were still subconfluent for 3 days (subc, lanes 4–6); growth on plastic in normal maintenance media until they were confluent for 5 days (conf, lanes 7–9); and growth on plastic in the presence of lactogenic hormones for 6 days (horm, lanes 10–12). The top panel indicates the Cripto expression in the populations. In the middle panel to compare the differentiation of cell populations, the 26 kDa β-casein and the 22 kDa milk proteins are indicated. α-Actinin staining was used as a control for equal loading of protein in the bottom panel

CID 9 cells *in vivo*

To determine if the over- or under-expression of Cr-1 had an effect on mammary gland development *in vivo*, we transferred CID 9 cells into the cleared fat pads of Balb/c mice. Unexpectedly, both Cr-1 sense-infected and uninfected CID 9 cells formed tumors in the transgenic fat pads, 5 to 6 weeks after transfer. We also tested the tumorigenic potential of the antisense Cripto expressing CID 9 cells. They remained tumorigenic, giving rise to tumors 6 weeks after insertion into the mammary fat pad of syngeneic Balb/c mice. The tumor growth rates were similar and the cells were histologically indistinguishable. Control mammary glands that were sham operated remained normal. Western blot analysis showed that approximately one-fifth the amount of Cripto was expressed in the tumors formed from antisense Cr-1 CID 9 cells compared to the parental CID 9 cells (data not shown). Therefore loss of antisense effect could not explain the unchanged tumorigenic potential of the CID 9 cells *in vivo*. We concluded that CID 9 cells were tumorigenic for reasons not relevant to Cripto expression.

We therefore turned to the parental cell line of the CID 9 cells, Comma-1D cells, to perform the same study, predicting that these cells may not be tumorigenic. Although uninfected Comma-1D cells transferred to cleared mammary fat pads produced no tumors, they did form hyperplastic outgrowths at the injection site in agreement with previous studies (Aguilar-Cordova *et al*, 1991). Comma-1D cells infected with the empty pGCEN or with the Cripto-expressing vector also formed hyperplastic outgrowths of similar sizes in numerous cases for each cell line. In contrast, Comma-1D cells infected with antisense Cr-1, were unable to grow in cleared fat pads, supporting

the finding that loss of Cr-1 decreases cell survival. This was retested in primary mammary cultures that were antisense Cr-1-infected and these also would not repopulate the cleared mammary fat pad, whereas uninfected cells would do so.

Discussion

The mammary gland is a highly complex system of interacting cell types. At birth, it contains at least three epithelial compartments: luminal epithelium, alveolar epithelium, and myoepithelium (Streuli *et al*, 1995). The mammary gland goes through four distinct postnatal developmental stages. Estrogen dependent ductal growth occurs from 5 to 8 weeks of age in the mouse when epithelial 'end buds' ramify from the nipple throughout the fatty mesenchyme creating a bush-like network of ducts. The onset of pregnancy initiates a second phase of extensive proliferation in which lobular-alveolar structures develop from the existing ductal system, in a process that is driven by pregnancy-induced hormones and estrogen (Nandi, 1958). It was from this stage that CID 9 cells were derived. The lobular system grows and differentiates to form alveoli in which milk protein synthesis occurs during lactation, the third phase of development. Following weaning of the young, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, a process called involution involving large scale apoptosis (Strange *et al*, 1995). Each phase of mammary gland development requires a specific combination of systemic hormones that presumably activate different combinations of locally acting factors. Signaling molecules that have been implicated in local actions include members of the EGF, Wnt, FGF and TGF-β families (Coleman and Daniel, 1990) (Snedeker *et al*, 1991). Each gene is expressed differentially during these developmental stages. We show here that Cr-1 is one of this group of genes, being strongly active during pregnancy, less active in lactation and switched off during involution of the mammary gland after pregnancy. This suggests both that Cr-1 expression is regulated by pregnancy hormones and that Cripto has a function during these stages of mammary gland development.

In this study we took advantage of a mammary cell subpopulation that mimics pregnant mammary glands in vivo. CID 9 cells allowed us to assay both growth and differentiation events in culture. We found that Cr-1 was expressed at higher levels when cells were stimulated to grow and differentiate to lactogenic phenotypes (in the presence of lactogenic hormones and extracellular matrix), similar to pregnant glands *in vivo* (Figure 1). This strengthens the hypothesis that these hormones up-regulate Cr-1 expression as they do amphiregulin (Martinez-Lacaci *et al*, 1995) and TGFα (Kenney *et al*, 1993; Reddy *et al*, 1994) in mammary tissue.

Kenney *et al*. (1995) showed by RT – PCR, the presence of Cr-1 mRNA in the virgin mouse mammary gland, although 24 or 26 kDa proteins were not observed using an anti-human Cr-1 antibody. We were able to detect low amounts of a Cr-1 protein of 26 kDa in virgin glands of 8 week and older mammary glands (Figure 1, lane 1) using a mouse-specific Cr-1 antibody. Twenty-four kDa is the predicted and expected size of the mouse secreted Cripto protein, demonstrated by Brandt *et al*. (1994) to be authentic Cr-1. In our analyses using immunoblotting of mammary gland tissues and cell lysates, we detected two Cr-1 proteins of 24 and 26 kDa (Figure 1) which are likely glycosylated or myristylated differently (Brandt *et al*, 1994). Cr-1 protein is expressed in undifferentiated F9 embryonal carcinoma (EC) cells as a protein of 24 kDa (Figure 1, lane 5) but is down-regulated in differentiated F9 cells, in agreement with others (Ciccodicola *et al*, 1989). CID 9 cells differentiate *in vitro* and we showed that Cr-1 has an inhibitory influence on differentiation. We demonstrated that proliferation increased and cell death decreased in CID 9 cells overexpressing Cr-1. Two separate assays, mammosphere formation and milk protein expression, showed that overexpression of Cr-1 led to decreased differentiation. In agreement with our data, a 47-mer, containing the EGF-like motif only of recombinant human CR-1 is able to stimulate proliferation and inhibit β-casein expression in mouse HC11 mammary epithelial cells (Kannan *et al*, 1997). It is generally accepted that proliferation rates are inversely related to differentiation, and we demonstrated here that Cr-1 stimulated the proliferation needed to bring the cells to the state required (equivalent to pregnancy) for differentiation (lactation) but inhibited the differentiation process itself.

We identified β-casein as a major product in differentiated CID 9 cells. Another milk protein of 22 kDa was also observed to be regulated independently of β-casein, a finding similar to that of Marte *et al*, (1995b) in neuregulin-treated HC11 cells which were also derived from COMMA-1D cells. The 22 kDa milk protein appeared to be specifically down regulated in CID 9 cells overexpressing Cr-1 when grown on an extracellular matrix. The nature of this protein and the mechanism of its regulation remains unknown.

How does the evidence presented here fit with the suggestion that Cr-1 is an oncogene? Cripto overexpression caused the cells to grow faster, to become less contact inhibited, allowed growth in domes, caused cells to survive clonal cell growth, and allowed anchorage independent growth (Figure 5, 6 and 7). Together these results suggest that Cr-1 overexpression leads to a transformed phenotype *in vitro* that would characterize Cr-1 as an oncogene. Moreover, when Cr-1 expression was reduced in cells that normally expressed it, they grew at one-third the rate of the control cells and became contact inhibited earlier and failed to grow anchorage independently or at clonal densities. This clearly suggests that Cr-1 is an autocrine growth factor for CID 9 cells. Our data and the NOG-8 results (Ciardiello *et al*, 1991) support the hypothesis that overexpression of Cr-1 leads to transformation and increased proliferation of normal mouse mammary epithelial cells. Our results also show that Cripto is required for normal growth and morphology of mammary cells during the pregnancy stage.

In contrast, *in vivo* tests do not lend support to a tumorigenic activity of Cr-1 in mammary cells. Although CID 9 cells are an excellent model for 'normal' mammary epithelial cell growth and differentiation *in vitro*, they proved to be tumorigenic when transplanted into syngeneic cleared

fat pads. In fact, control CID 9 cells (infected with empty vector) were able to grow in soft agar, suggesting they were already transformed. COMMA-1D cell studies were somewhat more revealing because when infected with the antisense Cr-1 retroviruses, they were unable to grow at clonal densities, indicating that these cells are much more sensitive to apoptosis in the absence of Cr-1. It is informative that NOG-8 mouse mammary cells even when over-expressing CR-1 do not form tumors in nude mice (Ciardiello *et al*, 1991), also suggesting that Cr-1 is not a tumor-inducing gene.

Some questions about Cr-1 need further study: What is the receptor for Cr-1 in CID 9 cells? What is the precise role of Cr-1 in human breast carcinoma, could Cr-1 act as a survival factor that allows the cells to escape apoptotic signals? High expression of Cr-1 in mouse and human embryonal carcinoma cells suggests that Cr-1 could play a role in teratocarcinoma formation (Baldassarre *et al*, 1997).

In summary, the results suggest that Cr-1 is positively regulated by lactogenic hormones and is important for mammary cell proliferation during the pregnancy stage of mammary gland development. Its function during pregnancy in the mammary gland may include differentiated cell renewal and survival but it is not directly involved in differentiation. Cr-1 also causes transformation *in vitro* and increases the rate of cell growth in mammary cells, a role suspected earlier because of its prevalent expression in breast and colon tumors. Using both *in vitro* and *in vivo* model systems we are addressing some of the questions concerning the regulation of Cr-1 and more specifically its role in mammary gland development and cancer.

Material and Methods

Cell culture

CID 9 cells were kindly provided by Dr. Mina Bissell (Lawrence Berkeley Laboratory, Berkeley, CA). COMMA-1D cells were provided by Dr. Dan Medina (Baylor College of Medicine, Houston, TX). Cells were maintained in 1 : 1 DMEM : Hams F12 Nutrient Mixture (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FBS and insulin (5 μg/ml). For differentiation, the cells were grown for 7 days in the presence of lactogenic hormones (insulin 5 μg/ml, hydrocortisone 1 μg/ml, and prolactin 3 μg/ml) on Matrigel (Collaborative Biomedical Products, Bedford, MA). The cells were plated in 1 : 1 DMEM : F12 Nutrient mixture, 2% fetal bovine serum (FBS), and lactogenic hormones at a density of 6×10^4 cells/cm^2. After 24 h the dishes were washed twice with PBS and fed with media containing no FBS but with lactogenic hormones.

Cell proliferation was quantified by determining formazan production from tetrazolium salt using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and a ELISA plate reader programmed to quantify absorbance at 570 nm and background at 630 nm. All assays were performed under conditions where the 570 nm absorbance readings were directly proportional to the number of cells/well. The anchorage independent growth assays were performed using a modification of a method described by Stoker *et al*, (1968). Cells were plated at a density of 4×10^4 cells per well of a 6 well plate in culture medium supplemented with 0.4% noble agar (Difco, Detroit, MI) over a lower layer of 0.3% agar, and allowed to grow for 21 days. Colonies were stained overnight with 0.05% p-iodonitrotetrazolium violet (Sigma Chemical Corp., St. Louis, MO), a vital stain that is taken up by mitochondria in cells.

Programmed cell death was quantified by (a) counting the the number of fragmented nuclei after staining logarithmically growing cultures with bisbenzamide (Sigma) and (b) *in situ* labeling of apoptosis induced DNA strand breaks (TUNEL assays) as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Greater than 2000 cells were examined per assay and each assay was repeated twice. The results were combined to give the results in Figure 6B.

In these assays, statistical analysis in the Macintosh Excel program was applied, using the students two-tailed *t* test.

Infections

The pGCEN vector was kindly provided by Dr. Richard Morgan (National Institutes of Health, Bethesda, MD) (Figure 2). The pGCEN neo retroviral vector expresses an inserted gene from the Moloney murine leukemia virus Long Terminal Repeat (LTR). An IRES sequence allows the expression of the neomycin resistance gene from the same promoter (Boris-Lawrie and Temin, 1993). The sense and antisense Cripto cDNA clones containing full length murine Cr-1 coding sequence was inserted into the *Xho*I site of the polylinker region in both sense and antisense orientations. PA317 producer cells (Miller and Buttimore, 1986) were transfected with 20 μg vector coprecipitated with calcium phosphate. Positive clones were selected with 800 μg/ml G418 after 5 days in culture. Supernatants free of G418 were collected and used immediately or aliquoted and stored at -70°C.

The CID 9 cells were infected with retroviral supernatant containing Cr-1 either in the sense or antisense orientation or the empty vector. The cells were plated at 8×10^5 cells per 100 cm^2 dish and the next day fed with 8 ml of viral supernatant (prefiltered through a 0.45 mm filter), 8 ml Hams F12 media, 4 μg/ml polybrene and 5 μg/ml insulin. After 24 h the infected cells were selected using G418 (400 μg/ml) in the media. Greater than 250 colonies were pooled and used in the studies as a mixed population. Because both Cr-1 sense and antisense sequences and the selectable marker neo are expressed from a single promoter using this vector, the cells were tested every few passages to determine that they maintained G418 resistance and hence Cripto expression.

Expression analysis

Mammary protein was obtained from mammary glands of staged mice. The tissues were homogenized in hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 μg/ml phenylmethylsulfonyl fluoride; 20 μg/ml aprotinin) and solubilized in sample buffer (Laemmli, 1970). Culture dishes were washed twice in phosphate buffered saline (PBS) and lysed in sample buffer. Cells grown on the extracellular matrix were first treated with dispase (Collaborative Biomedical Products, Bedford, MA) for 1 h to dissolve the matrix and then washed and lysed as above. Equal amounts of protein were electrophoresed on a 15% SDS–PAGE gel and electrotransfered to Immobilon membranes (Millipore Corporation, Bedford, MA). Western blot analysis was performed and visualized using the ECL detection system (Amersham Corp., Little Chalfont, UK). Indirect immunofluorescence assays were done on fixed cell monolayers using monoclonal antibodies to pan-keratin or vimentin (Sigma Chemical Corp., St Louis, MO) with FITC-labeled rabbit anti mouse Ig as secondary antibodies.

The rabbit polyclonal antibody was raised against a murine Cripto peptide, amino acid sequence 26 to 39, RDLAIRDNSIWDQK. The antimouse milk serum was a generous gift from Dr N Hynes (Friedrich Miescher Institute, Basel Switzerland). This antiserum recognizes several milk protein including β-casein and the 22 kDa protein (Marte *et al*, 1995b). Sheep anti-mouse casein antibody was kindly supplied by Dr B Vonderhaar (NCI). A rabbit polyclonal antibody to rat α-actinin was a gift from Dr J Singer (UC San Diego, CA) and served as a control for equal protein loading on the gel.

Mammary fat pad transplants

Mice were anesthetized with avertin. Mammary gland 'clearing' was performed on the right inguinal #4 fat pads of mice at 3 to 4 weeks of age as described previously (Deome *et al*, 1959; Faulkin and Deome, 1960). In essence, the nipple and primitive adjacent mammary epithelial tissue were excised while the remaining fat pad provided the region for growth of transplanted cells. The CID 9 cells were washed in serum-free medium and injected into the fat pads at approximately 1×10^5 cells per fat pad in 5 to 10 μl volumes (Edwards *et al*, 1988). The skin was sutured, and mice maintained for 6 weeks to allow the transplanted cells to grow in the fat pad. Glands or tissues were dissected out, divided into portions and frozen for later analysis.

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