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Cripto-1 (Cr-1) and Amphiregulin (Ar) are two EGF-like growth factors that occur at higher levels in breast cancer cells and tissues. We have shown that they occur in the late stages of mammary cell proliferation in virgin, pregnant and lactating mouse mammary glands. In order to test the roles of Cr-1, we infected normal mouse mammary cells with retroviruses over-expressing Cr-1, or an antisense version, in order to inhibit the production of this polypeptide. Cr-1 stimulated proliferation but inhibited differentiation (lactation). Reduction of Cr-1 decreased growth rate and increased apoptosis, but the cells were able to differentiate. Cr-1 overexpression appears not to be tumorigenic. The main approach for Ar will be to make transgenic mice overexpressing Ar in the mammary glands.

We completely inactivated the Cr-1 genes in order to produce mice that expressed no Cr-1 in mammary glands as well as in all other tissues. To this end, we inactivated Cr-1 in embryo stem (ES) cells by homologous recombination and produced Cr-1 gene-targeted mice. Studies on these Cr-1(-/-) cells showed that ES cell differentiation to cardiac myocytes was prevented. We have also produced "knockout" animals with Cr-1 null ES cells and shown that Cr-1 is essential for embryonic development. We made chimeric animals that will be useful for obtaining supporting data by isolating mammary cells that are Cr-1 null.

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E. D. Adamson 10/13/97

PI - Signature Date

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(5) INTRODUCTION

The expression of the EGF-like ligands, Amphiregulin (Ar) and Cripto-1 (Cr-1) gene products as revealed by immunocytochemistry, was thought to be restricted to tumors of the breast, ovary and a few other tissues, with little or no expression in adjacent normal tissues. A new study in the literature indicates that Cr-1 is overexpressed in 100% of non-seminomatous germ cell tumors (1). However, there are some situations where Ar and Cr-1 expression do occur normally, and their activities and roles are of interest because we may start to understand the conditions that deregulate their expression or their activities.

The epidermal growth factor (EGF) receptor is known to bind and respond to Ar, but Ar has a distinctive effect compared to the other 5 ligands for cells that respond to its signal. Ar is known to be a product of many human breast cancer cell lines, but whether it has a role in the tumorigenic process is one question that we are addressing. In contrast, Cr-1 is an orphan ligand, with no known receptor, since the truncation of the sequence within the EGF-like domain makes this ligand unable to bind to the EGF receptor. Data from the laboratory of Dr. M. Kirschner, indicates that Cripto-like ligands produced in Xenopus laevis (frog) may be related to the fibroblast growth factor (FGF) family of ligands and may bind to an FGF receptor molecule but this has not yet been established (4). New data from the laboratory of Dr P. Leder, indicated that an additional Cr-1-related molecule, *Cryptic*, is produced in mouse. Cryptic has a different expression range than Cr-1, but is also expressed in the postimplantation embryo. Thus Cr-1 is a now one member of a family of genes with the proposed name CFC (5)

The main theme of the present research is based on findings that gene products that have EGF-like sequence domains can act by a) stimulating autocrine or paracrine growth, and b) stimulating the synthesis of some of the other members of the ligand family and c) inducing the prolonged expression of the epidermal growth factor receptor (EGFR). These events are hypothesized to lead to stimulation of growth and to conditions that are conducive to genetic changes that may lead to cancer. The question that we are addressing is whether Ar and Cr-1 can initiate the tumorigenic process in mammary gland.

We have shown that Ar and Cr-1 are produced during the normal postnatal development of the mammary gland in mouse (2). These growth factors are just perceptible among the proteins in the 12 week old mammary gland (using immunoblotting, immunocytochemistry and reverse transcription polymerase chain reaction, RT-PCR), but greatly increase during pregnancy and are at the highest level in late pregnant and early lactating glands.

We have explored the roles of Cr-1 in mammary glands by over-expressing and by inhibiting Cr-1 expression in a normal mouse mammary cell line, CID 9 (this work has been submitted for publication and a copy is in the Appendix). In order to completely negate the expression of Cr-1 in animals, it is necessary to target the gene with a gene disrupting vector using homologous recombination. We have made great strides in the process towards the inactivation of the Cr-1 gene in embryo stem (ES) cells, in embryonal carcinoma (EC, F9) cells and in animals (see below).

We have also collaborated with Dr Lynn Wiley at the University of California at Davis (UCD) to show that Ar is produced by preimplantation embryos where it also appears to assist in the process of growth and development of the blastocyst (**publication 1**). Dr Wiley, her postdoctoral fellow and I wrote a review earlier, on the topic of EGF receptor activites in the preimplantation embryo for BioEssays (6). Dr Wiley and I have also written an extensive review on the broad topic of EGFR activities and this is in press in Current Topics in Dev. Biol. **(publication 3)**.

In summary, we have had to alter the tasks initially outlined partly because Dr Kenney left the lab and took some parts of the work with him in his new position at Georgetown University Cancer Center. Dr Kenney has attempted the over-expression of both Ar and Cr-1 in primary mammary epithelium cells, and the transplantation of these cells into syngeneic mammary fat pads. He has one paper published in

December on the roles of amphiregulin in mammary development and cancer (3). Another paper involving Cripto-1 is in press. In order to avoid repetition, we have followed essentially the same tasks using the same technique of mammary cell infection and transplantation, but using a normal mammary cell line instead of primary cell cultures. The two studies also differ in that the retrovirus that we use has a different promoter. The chief difference in the scope of the work is that Dr Kenney did not attempt to make *under-expressing* mammary cells because he did not make an antisense version of his vector, and so could not show that Cr-1 has *autocrine* growth effects, but he has results that indicate that both Cr-1 and Ar have proliferative effects on primary mammary cells after transplantation. The Cr-1 work is not yet published and our knowledge of Dr Kenney's results is incomplete, since we no longer communicate.

The initial SOW was modified in order to respond to these changes in personnel, and to tackle the concept of gene inhibition with a more robust plan. The changes of plan were described in previous years reports. Other changes in our detailed plans occurred in response to our own findings and the results of others. The overall aims are important and remain the same.

(6) BODY

i) Project to over- and under-express Cr in mammary cells.

a) *Methods.* The principal method to reveal the biological role of a gene product is to manipulate its expression in cells or in the whole animal. We can report significant progress in both these approaches. Dr Christine Niemeyer constructed a retroviral expression vector that can be used to infect mouse cells (CID 9 mammary cells) so that they over-express Cr-1 in one population, or have reduced Cr-1 expression in another. The control population was infected with an "empty" retrovirus. This is a necessary control to indicate if the infection produced an effect independently of the gene insert.

b) The *results* reported last year were extended, and are summarized as follows. Cripto 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. Our results showed that aberrant expression of Cripto affected the growth, morphology, and differentiation of these cells. Exogenous mouse Cripto 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 overexpressing Cripto grew anchorage-independently and did not differentiate efficiently. Infection of CID 9 cells with a Cripto antisense vector caused these cells to change in morphology, to grow slowly and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cripto is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the stimulation of proliferation at the expense of breast cell differentiation by Cripto. The main question that we wished to address is whether Cr-1transforms mammary cells, because the results in vitro suggested that it did.

When these Cr-1-under and over-expressing CID-9 cells were transplanted into mammary fat pads of syngeneic mice, they all produced mammary tumors, although, the literature indicated that the cells were, at one point, *not* tumorigenic. The disadvantage of using cell lines is that they change with increasing passage number. We were aware of this possibility and started with low passage cells specially obtained from Dr Mina Bissell.

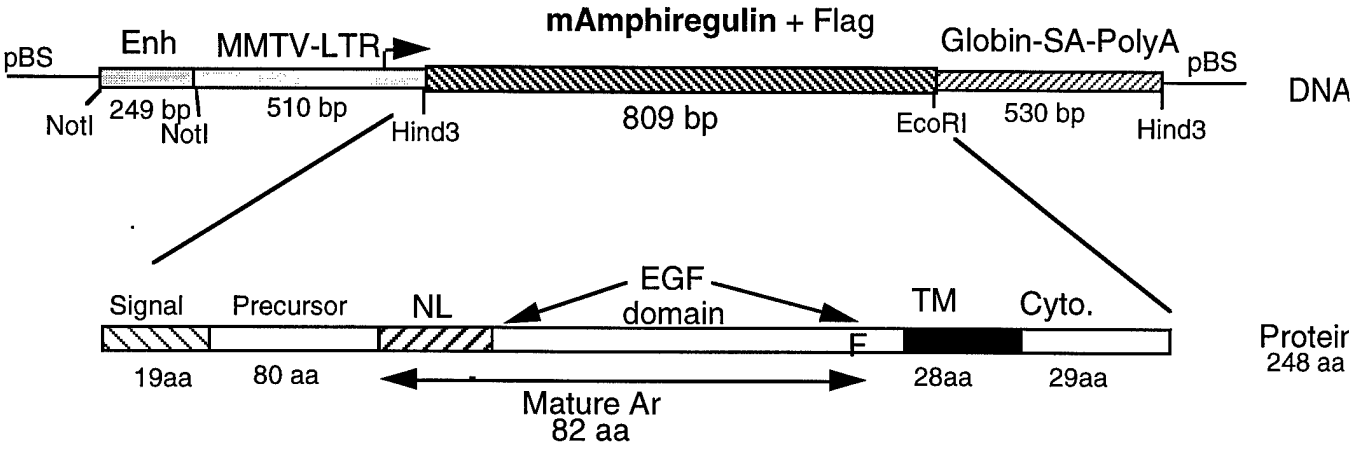
We next obtained a similar mammary cell line, COMMA-D that is less tumorigenic and gave no tumors after transplantation but did produce hyperplasia in 15 out of 18 cases, whether the cells were wild-type controls or over-expressing Cr-1. Interestingly, COMMA-D cells expressing little or no Cr-1 due to antisense expression, did not grow at all, either normally or abnormally. One new conclusion was possible based on the results in these cells: the removal of expression of Cr-1 in COMMA-D mammary epithelial cells counteracts their inherent growth capacity after transplantation. Furthermore, we also showed that the removal of Cr-1 from mammary cells increases the normally low level of apoptosis three-fold. This increase in programmed cell death explains why mammary cells lacking Cr-1 did not survive after transplantion. This result confirmed the previous finding that Cr-1 is necessary for normal mammary gland growth and development.

In summary, Cr-1 is required for normal mammary cell survival, growth and for epithelium development, but probably does not transorm mammary cells. Although not proven by our results, the data suggest that Cr-1 is not an initiator of the tumorigenic process. The manuscript that we reported earlier has now been modified to include the new data and has been submitted to Cell Death and Differentiation (**publication 2**).

ii) Project to over-express Ar in mammary epithelium.

a) *Methods* are similar to those described for Cr-1. Figure 1:

Mammary expression vector for Ar in TGM



NL = nuclear localization and heparin binding domains

F = Flag Epitope

The experimental approaches originally described, are in progress also for Ar, but modified by using COMMA-D or the related HC11 normal mammary epithelial cells, and are being performed by Dr Bradley Spencer-Dene. We have prepared Ar expressing retroviruses and plasmid vectors to choose the best expression vector. Dr Spencer-Dene has prepared a plasmid expression vector (see **Fig. 1** above) to over-express Ar using an inducible promoter, MMTV, which is induced by the glucocorticoid analog dexamethasone, as well as lactogenic hormones. The additional feature that we are including is an epitope to a tag "FLAG", that can theoretically be detected using a commercial anti-FLAG antibody. This allows us to assay the expression of the exogenous vector in cells that also express endogenous Ar. However, it turns out that the epitope is not detectable in cells transfected with the vector and, therefore, must be masked during folding of the protein. The antisense version of this has not yet been successfully made, after several attempts and we have decided to go ahead with the sense version alone.

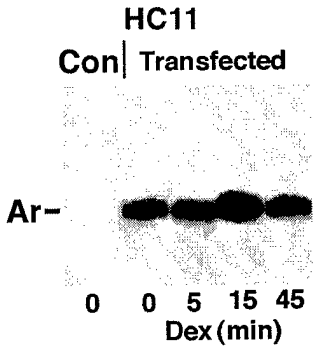


Figure 2 Mouse mammary epithelial cell line, HC11 were transfected or not (Con) with MMTV-neo-Ar plasmid. G418 resistant cells were selected for 2 weeks and the population was taested for Ar expression by immunoblotting. Four duplicate dishes were stimulated for 0, 5, 15 and 45 min with dexamethasone to induce the MMTV promoter. Ar expression was maximal at 15 min after addition of Dex.

b) The *results* of the transfection of the vector into HC11 cells is shown in the **Figure 2.** The expression is x-fold increased and is y-fold induced in the presence of 2μM dexamethasone for 15 min. This is our first success in amplifying the production of Ar in mammary cells. It is possible that there is feedback inhibition because in CID9 cells, the expression of Ar is already high and is only slightly increased by transfection of the same vector that works in HC11 cells. We are working on the vector to maximize its "strength".

iii) Project to make antibodies to Cr-1 and Ar.

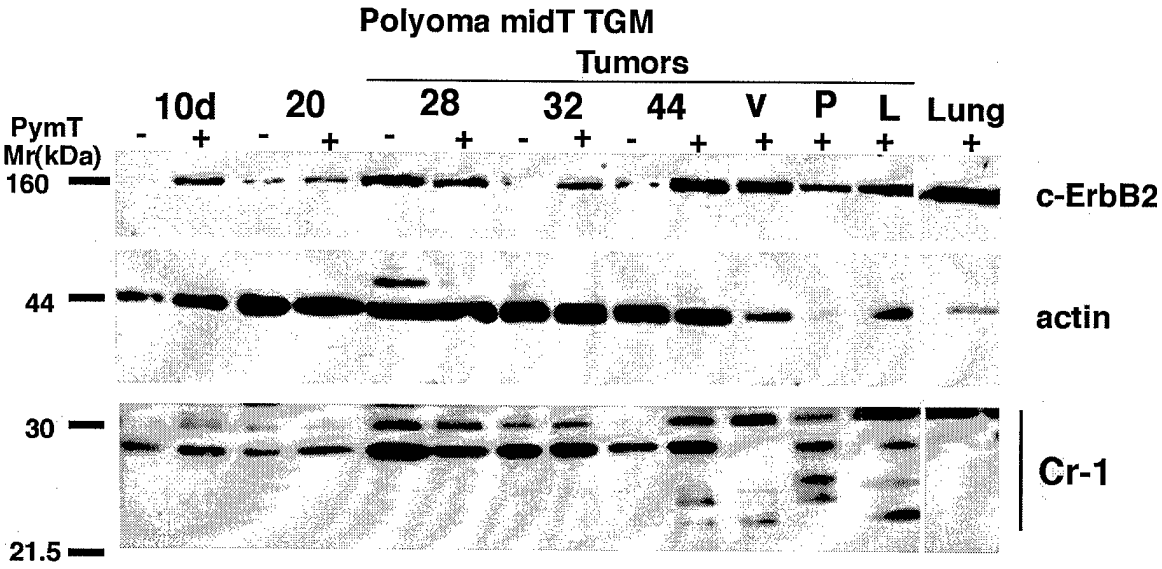
We have now successfully raised rabbit antibodies to Ar. a) *Methods.* We purified the original 17-mer peptide by HPLC and have made specially formulated immunogen as a mixture of 2 types of crosslinking with carrier protein, in order to increase the immunogenicity of Ar. We need the antibody to assay the level of expression of the Ar protein product in our studies to over- and under-express Ar in mammary cells. The results in Figure 2 were obtained with the new antibody.

b) *Note.* We are continuing to use the antiserum raised by Dr Persico to a peptide within the mouse Cr-1 sequence. This gives a large number of high Mol. Wt bands on gels that we cannot account for, so this remains a significant problem. Expression studies can be performed by assaying the level of mRNA, but will not answer the question of whether the mRNA is translated and whether the role of the gene product is really being tested.

iv) Project to determine the time course of the expression of Cr-1 and Ar during tumorigenic progression.

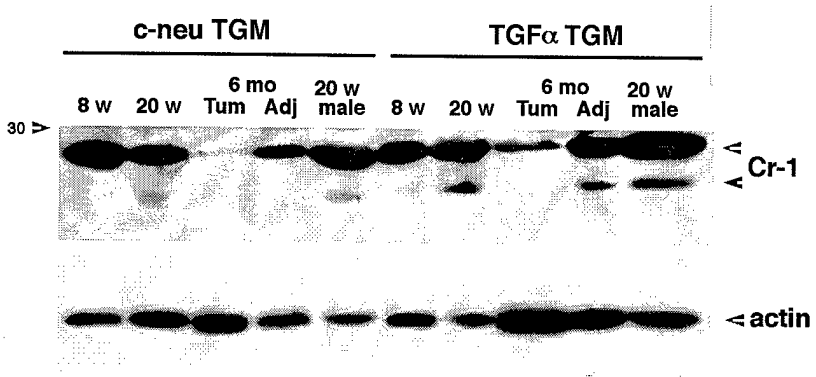
a) *Methods.* Transgenic mice were provided by Dr W. Muller, or were purchased from the Jackson lab. We have examined the mammary glands before and during the tumorigenic event in several transgenic mouse (TGM) models using Western blotting. One TGM line was made by the insertion of the Polyoma middle T antigen behind the mouse mammary tumor virus (MMTV) LTR originated by Wm Muller. We have also obtained TGM from the Jackson lab that overexpress MMTV-c-neu (ErbB2), and TGM that overexpress MMTV-TGFα in mammary glands. The c-neu-expressing mice give rise to tumors after about 16 weeks of age, but for the TGFα TGM, tumors only arise after 12 months of age.

b) *Results.* i) The females of PyT TGM develop multifocal mammary tumors at around 20 to 34 days of age, while tumorigenesis in the males takes longer. We found that both Ar and Cr-1 are over-expressed in the large late tumors, and we have almost completed a time course to determine if either of these proteins could act as markers for an early or a specific stage of mammary tumorigenesis. A preliminary result is shown in **Figure 3**. These have be repeated with more equal loading of samples on the gels.



The result so far indicates that Ar but not Cr-1 is expressed by the mammary tissue before the appearance of PymT tumors starting at around day 28. However, the presence of Ar is equal in both the non-tumor as in the tumor-expressing mammary glands (data not shown). More data are needed to quanitify this result. In addition the expression of ErbB2 but not ErbB1 (EGFR) is induced in the mid sized tumors in mammary gland starting on the 32nd day (Fig. 3). In the tumors in virgin mice (V) the levels of Cr-1, Ar and ErbB2 are lower than in pregnant mice (P) and in lactating mice (L), suggesting that steroid hormones regulate production.

ii) In tumors of the ErbB2 (c-neu) TGM small tumors arose but expressed *lower* levels of Cr-1 than in normal mammary glands **(Figure 4, lane 3).**



We are also following the expression of EGFR and ErbB2 during this time course because Ar can produce signals from both these receptor kinases. We obtained only "calcified tumors" from one TGFα TGM (at 6 months of age) that were in the sebaceous glands and these did not express either Ar or Cr-1 (Fig. 4, lane 8, shows that TGFα expression was reduced in these nodules).

This project is still in progress, to repeat the findings and to include assays of the expression of ErbB2 and EGFR, during the time course. The long-term aim is to make or obtain mice with different capabilities in mammary tumor formation and mammary cells with different production levels of of Ar and Cr-1, in order to collect further data to determine if there is a correlation.

We will then investigate the effects of cross transplantation of mammary epithelial cells into the fat pads of tumorigenic and normal mice to investigate the role of Cr-1 and Ar in the interaction of stroma with mammary epithelium. Making mice that express no Cr-1 in mammary glands was part of this aim. In the coming years, we will cross-breed transgenic mice with tumorigenic potential in the mammary glands that are available, to determine the conditions and levels of Ar and Cr-1 expression that give rise to hyperplasia, dysplasia, carcinoma is situ and mammary carcinoma. For example, we are planning to make EGFR-overexpressing TGM using MMTV-EGFR (funded by different grant), and cross breed this mouse with MMTV-Ar TGM to determine if there is synergism and earlier tumor formation.

v) Project to make MMTV-Ar transgenic mice.

a) *Methods.* We have decided that the most urgent and unique study not so far performed or described in the literature is the over-expression of Ar in transgenic animals. The procedures are performed by the transgenic mouse facility at the Institute. We will overexpress Ar specifically in mammary glands of TGM, using MMTV-Ar expression vector. This will be the major aim for the coming year. The construct is ready (Figure 1) but is being further tested to determine if the addition of an enhancer from the MMTV LTR is useful in boosting the expression of Ar in mammary cells.

vi). Progress report on the inactivation of Cr-1 in ES cells and in vivo.

Methods. Dr Chunhui Xu in the laboratory has made a targeting vector for homologous recombination and targeting, that recognizes the mouse Cr-1 gene. She has used the vector to perform the technique of homologous recombination in order to inactivate the Cr-1 gene in ES cells. Standard procedures were used to perform the knockout in ES cells and these were done by Dr Xu. The microinjection of the resulting ES cells into blastocysts was performed by the transgenic mouse facility personnel. There are 2 parts to this study:-

a) Two of the ES cell clones that were assayed and found to contain a single inactivated Cr-1 gene were microinjected into mouse blastocysts (a pay back service at the Burnham Insititute) following published procedures for making "knockout " mice. We have 20 male chimeras and these produced heterozygous Cr-1 knockout mice. We bred these mice and produced Cr-1 (-/-) mice without any Cr-1 expression. *Results.* The homozygous embryos died at gestation day E7 to E9, due to the inability of the mesoderm and ectoderm to properly undergo morphogenesis. As a result there is no recognizable development after the initial formation of the neuroepithelium, there is no further differentiation of the cardiac mesoderm and no beating heart. The visceral yolk sac alone continued to develop up to E10 but there were no embryos within the yolk sacs. Further assays are needed to determine the major target tissue taht fails to progress due to the lack of Cr-1.

b) The inactivation of the second Cr-1 allele in ES cells has produced cells with a major defect that is only obvious when the cells are cultured under conditions that lead to their differentiation. One of the most frequent types of cell produced by differentiated ES cells is the beating cardiac myocyte which are seen on the 7 th day and beyond. The ES-Cr(-/-) cells were unable to differentiate into cardiac myocytes. No beating was seen in 21 days of culture and no cardiac myosin protein was detected in immunoblots. This was specific because alphafetoprotein (AFP) was produced in normal amounts in all ES cell types, Cr-1(+/+), (+/-) and (-/-). AFP is another product secreted from differentiated ES cells. Moreover, the effect was dose dependent, because the (+/-) ES cells were 2 days delayed in their ability to give beating heart muscle. In addition, we "rescued" the mutant behavior by transfecting the Cr-1 null ES cells with an expression vector for Cr-1. In those cells only and not the antisense transfected cells, cardiac differentiation was restored. We tested for all the early markers of differentiation to the cardiac lineage and found that Cr-1 null cells are unable to switch on any of the cardiac myosin genes. They do express all of the transcription factors that are known to be in the cardiac pathway, and so we are very excited that Cr-1 appears to be a major switch gene for the differentiation of cardiac muscle. Importantly, skeletal muscle was not affected, and Cr-1 null cells produced striated muscle myotubes in culture. We have submitted a manuscript describing these results to Developmental Biology (**publication 4,** copies in the Appendix).

vii) Rescue of the lethality due to Cr-1 abrogation using chimeric animal constructs.

a) *Methods.* We used our ES(KO)2 cells to make chimeric animals by their microinjection into normal C57Bl6 blastocyst embryos. The idea is that, if null cells are acting autonomously and are unable to take part in the development of a specific tissue, such as the heart, the resulting chimeric animals will have only normal cells in the heart and Cr-1-null cells will be excluded.

b) *Results.* The result was that the Cr-1-null ES cells took part in the development of every tissue in the resulting adult animals, and these were alive and well. Our conclusion was that since Cr-1 is produced as a soluble protein, it was able to rescue the null phenotype of the null component of the mice so that it was able to take part in development. We proved by this result that Cr-1 is a soluble, diffusible protein with a range of distal activities that compensate for inactive Cr-1 genes.

We plan on using these mice for the preparation of totally Cr-1 null primary mammary (and other) cell cultures. This can be done because the null cells are neomycin resistant, while the normal cells are not. We will then test their ability to grow in monolayers, in aggregates in soft agar and in colonies to determine if they have reduced survival as our data on antisense expressing cells suggest. We will also test their ability to reconstitute the mammary gland by transplantation into cleared mammary fat pads of syngeneic animals. It is possible that these cells will not survive at all in the absemce of Cr-1 expression. This approach is the cleanest way to demonstrate that characteristic.

(7) CONCLUSIONS

Cripto-1.

1. Cr-1 protein contributes to the growth and development of normal mouse mammary epithelial cells. Cr-1 production is up-regulated by pregnancy hormones, while regressing mammary glands produce no Cr-1. The data indicate that Cr-1 plays a role in the events leading to differentiation (lactation) but **not** in differentiation itself. Reduction of Cr-1 levels inhibits growth in monolayers and in soft agar. Cr-1

reduction increases apoptosis, but does not affect their ability to differentiate as shown by the production of milk proteins. Cr-1 is a survival factor for mammary epithelial cells.

2. Data from homologous recombination studies (gene targeting) in ES and EC cells indicates that Cr-1 is needed to maintain the high rate of proliferation of undifferentiated EC cells.

3. Cr-1 plays a role in the events leading to cardiac cell differentiation at a stage before the synthesis of myosin. The loss of Cr-1 does not affect the ability of differentiating ES cells to produce a large number of other markers of differentiation.

4. Cr-1 is necessary for embryonic development from the E7 stage, and absence leads to lethality soon after.

5. Cr-1 is probably *not* a transforming growth factor for mammary cells, and is *not* an early marker of the tumorigenic process.

Amphiregulin.

1. Ar is expressed in early mouse embryos as early as the 8-cell stage, in the inner cell mass and the trophoblast cells of the blastocyst. It plays a part in the development of the preimplantation embryo based on inhibition of cell numbers in embryos incubated in vitro with antisense Ar oligonucleotides.

2. Ar is expressed in the late stages of postnatal mammary gland development. It is produced maximally in pregnant glands and is less prominent in lactating glands. Ar is an autocrine growth factor for mammary cell proliferation.

3. Ar production is increased in mammary glands from MMTV-PymidT mice before the production of overt tumors. More evidence is needed to order to identify this growth factor as a contributor to the tumorigenic process, or as a marker or precancerous conditions.

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Completed tasks for Cr-1

We have completed tasks 1-3 of the original SOW (omitting TGFα as explained earlier).
We have not attempted to do the time points indicated in task 4 because the cells were already rapidly tumorigenic, even without further expression of Cr-1.
We have completed task 5 for the current CID9 cells and COMMA-D cells, which are less tumorigenic.
We have totally abrogated the activity of Cripto genes in ES and F9 EC cells and analyzed the effects.

We now have Cr-1(+/-) mice which are viable and healthy. We bred these mice to produce homozygous Cr-1(-/-) embryos that die on gestation day 7 to 8. Therefore Cr-1 is important in early postimplantation development.

Completed tasks for Ar

We have succeeded in making a rabbit polyclonal antibody to Ar, that works in immunoblotting (Westerns).

We have almost completed the construction and testing of a mammary specific expression vector (MMTV-LTR-Ar) to over-express Ar in mammary cells and glands.

Statement of Work

1. Tasks 1-3 for Ar will be performed using CID9 cells (which express a high endogenous level) and HC11 mouse mammary epithelial cell line that expresses very low levels of Ar . This will test the effect of over-expression of Ar in mammary epithelial cells and their ability to grow and differentiate normally. We will test their ability to grow in soft agar and if this is positive, we will test their tumorigenic potential as a function of the level of Ar expressed.

2. We will microinject the MMTV-Ar construct into mouse eggs to make transgenic mice that over-express Ar in the mammary glands. We will analyze the mice for developmental effects on all stages of mammary development, and continue to observe the mice into late adult life before and after pregnancy to determine if over-expression leads to tumor formation. The TGM will be bred to MMTV-TGFα TGM to determine if the two ligands act synergistically or additively, or, to determine if no further increase in tumorigenicity develops by doublly overexpressing mice.

3. The analyses of transgenic mice over-expressing a) polyoma middleT (MMTV-PyT), b) MMTV-c-neu, c) MMTV-TGFα for the levels of expression of Ar, Cr-1, EGFR and ErbB2 levels during a time course will be completed and a paper written describing the results.

4. Transplantation of mammary cells from MMTV-PyT mice into normal mice and the reverse will be performed (original tasks 6, 7 and 8).

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APPENDIX

PUBLICATIONS

1. Tsark, E., Adamson, E.D., Withers, G.E. and Wiley, L.M. (1996) Expression and function of amphiregulin during murine preimplantation development. Mol. Devel. Reprod. 47: 271-283.

2. Niemeyer, C.C., Persico, M.G. and Adamson, E.D. (1996) Cripto: roles in mammary cell growth survival, differentiation and transformation (under revision for Cell Death and Differentiation).

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MOLECULAR REPRODUCTION AND DEVELOPMENT 47:271–283 (1997)

Expression and Function of Amphiregulin During Murine Preimplantation Development

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ABSTRACT Amphiregulin (Ar) is an EGF receptor ligand that functions to modulate the growth of both normal and malignant epithelial cells. We asked whether mouse preimplantation embryos express Ar, and if so, what the function of Ar is during preimplantation development. We used RT-PCR to show expression of Ar mRNA in mouse blastocysts, and using a polyclonal anti-Ar antibody and indirect immunofluorescence, we detected the presence of Ar protein in morula- and blastocyst-stage embryos. Ar protein was present in both the cytoplasm and nucleus in both morulae- and blastocyst-stage embryos, which is similar to Ar distribution in other cell types. Embryos cultured in Ar developed into blastocysts more quickly and also exhibited increased cell numbers compared to control embryos. In addition, 4-cell stage embryos cultured in an antisense Ar phosphorothioate-modified oligodeoxynucleotide (S-oligo) for 48 hr exhibited slower rates of blastocyst formation and reduced embryo cell numbers compared to embryos exposed to a random control S-oligo. TGF-α significantly improved blastocyst formation, but not cell numbers, for embryos cultured in the antisense Ar S-oligo. From these observations, we propose that Ar may function as an autocrine growth factor for mouse preimplantation embryos by promoting blastocyst formation and embryo cell number. We also propose that blastocyst formation is stimulated by Ar and TGF-α, while Ar appears to exert a greater stimulatory effect on cell proliferation than does TGF-α in these embryos. *Mol. Reprod. Dev. 47:271–283, 1997.* © 1997 Wiley-Liss, Inc.

Key Words: autocrine; preimplantation embryo; RT-PCR; TGF-α; amphiregulin

INTRODUCTION

The murine preimplantation embryo expresses several growth factor receptors and their ligands that are thought to account for the ability of the embryo to develop in vitro without added growth factors (for reviews see Wiley et al., 1995; Schultz et al., 1993; Heyner et al., 1993; Adamson, 1993; Pampfer et al., 1991). These embryo-produced growth factors and growth factor receptors appear to promote blastocyst development by stimulating both cell proliferation and development of the trophectoderm, a fluid-transporting epithelium responsible for blastocoele formation (Paria and Dey, 1990; Dardik and Schultz, 1991; Rappolee et al., 1992; Brice et al., 1993).

Amphiregulin (Ar) differs from other EGF receptor ligands in that it contains 2 putative nuclear localization sequences, contained in a basic, hydrophilic NH_2-terminal region, which are believed to target Ar to the nucleus of several cell types (Shoyab et al., 1989; Johnson et al., 1991; Johnson et al., 1992; Ebert et al., 1994; Normanno et al., 1994; Akagi et al., 1995). However, Ar has many similarities with other EGF receptor ligands. For example, Ar binds to and induces autophosphorylation of the EGF receptor and $p185^{erbB2}$ (Johnson et al., 1993) and is proteolytically cleaved from a larger transmembrane precursor that resembles a cell surface receptor (Plowman et al., 1990). The sequence corresponding to the mature, soluble form of the growth factor is located in the extracellular domain of this precursor, and its 40 amino acid COOH-terminal region contains 38% and 32% sequence homology to EGF and TGF-α, respectively. Ar also contains the 6 cysteine residues that interact to form 3 intramolecular disulfide bonds characteristic of EGF-like proteins.

In the present study, we asked two questions. First, do murine preimplantation embryos express Ar? Second, if embryos express Ar, what is the function of Ar during murine preimplantation development? In contrast to a previous report (Johnson et al., 1994), our findings suggest that murine preimplantation embryos express both the mRNA and protein for Ar and that it may be functioning as an autocrine growth factor by stimulating embryo cell proliferation and trophectoderm differentiation.

MATERIALS AND METHODS

Embryos

Eight-to-twelve-week-old females of the CD-1 strain (Charles River, MI) were injected with 5 IU of pregnant

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mare serum gonadotropin (PMSG, Sigma) and then, 48 hr later, with 5 IU of human chorionic gonadotropin (hCG, Sigma) to induce superovulation and immediately paired with CD-1 males. The females were sacrificed 48 hr later by cervical dislocation and 4-cell embryos were flushed from the oviducts with modified Hank's basal salt solution (Goldstein et al., 1975). The embryos were transferred to 20 µl drops of modified (Wiley et al., 1986) T6 medium (Quinn and Whittingham et al., 1982) overlaid with paraffin oil (Dow Chemical Co.), placed in a water-jacketed incubator set at 37°C, and filled with saturated 5% CO_2 in air.

Generation and Characterization of the Polyclonal Anti-Ar Antibody

A peptide corresponding to residues 118 to 133 in mouse Ar with an added tyrosine residue to facilitate linking (Y-RKKKGGKNGKGRRNKK) was synthesized by the protein chemistry laboratory of the Burnham Institute. This peptide corresponds to a similar sequence used to make antibodies to human Ar (Ab-2, Johnson et al., 1993b), except that there were two amino acid differences between the human and mouse sequences. The peptide was linked to Keyhole limpet hemocyanin (KLH) protein to produce the immunogen. Five hundred µg peptide-linked KLH was homogenized with Freund's complete adjuvant for the first injection (subcutaneous) into 2 rabbits. Subsequently, 250 µg booster injections in Freund's incomplete adjuvant were made. The second to fifth bleeds were reactive to the peptide and to the protein in immunoblotting assays performed on pregnant mammary gland tissues lysates. Polypeptides of about 20 and 28 kDa were observed (data not shown) similar to as in published results (Kenney et al., 1995, 1996). Immune IgG was affinity purified from antisera on peptide linked to Sepharose, eluted with 0.1 glycine-HCl, pH 2.5, immediately neutralized, and dialyzed. Aliquots of affinity purified IgG were stored frozen at −70°C.

Indirect Immunofluorescence Assay for Detection of Ar Protein

Morulae and blastocysts were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and subsequently permeabilized with 0.05% Triton X-100 in PBS for 2 min. Triton X-100 has been shown to enhance the staining of Ar in the nucleus of cells (Akagi et al., 1995; Johnson et al., 1991). Fixed permeabilized embryos were incubated in normal goat serum (undiluted) overnight at 4°C to block nonspecific binding sites and the zonae pellucidae were removed by brief exposure to acidic PBS (pH 2.5). Zona-free embryos were incubated for 30 min in the anti-Ar IgG at a concentration of 40 µg/ml. Specificity of the anti-Ar IgG was tested by preabsorbing the IgG with the immunizing peptide. Distribution of bound Ar IgG was visualized using rhodamine-conjugated goat anti-rabbit IgG (1:100) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After rinsing with BSS/BSA, the embryos were transferred to a drop of BSS/BSA on a coverslip and immediately observed with an inverted phase contrast microscope fitted with epifluorescence illumination.

RT-PCR

Using the murine Ar cDNA sequence (Das et al., 1995), 2 PCR primer pairs were designed. Primer pair no. 1 consisted of a forward (upper) primer with the sequence 5′-CAG GGG ACT ACG ACT ACT CAG A-3′ and a reverse (lower) primer with the sequence 5′-GAT AAC GAT GCC GAT GCC AAT A-3′. Primer pair no. 2 consisted of a forward primer with the sequence 5′-AGT GCT GTT GCT GCT GGT CTT AG-3′ and the same reverse primer as primer pair no. 1. Primer pair no. 1 gives a PCR product that is 434 bp in length and spans exons 2–4, whereas primer pair no. 2 gives a PCR product that is 613 bp in length and spans exons 1–4. The primer pairs were designed to span introns so we could distinguish between products amplified from cDNA (reverse-transcribed from mRNA) and those amplified from contaminating genomic DNA.

The reverse primer was used for reverse transcription. In a thin-walled PCR reaction tube the following were placed: 100 mouse blastocysts transferred in approximately 2 µl of culture media, 2 µl of 5% NP-40, 5 µl sterile water, 2 µl reverse primer (0.5 µg/µl), and 1 µl RNAsin (40 units/µl, Promega). This was then heated to 65°C for 5 min, centrifuged quickly, then cooled to 4°C for another 5 min. To this mixture, the following was added: 5 µl of dNTPs (5mM each, Promega), 5 µl 5× MMLV-RT first strand buffer (Gibco BRL), 2.5 µl 0.1 M DTT (Gibco BRL), and 1 µl MMLV-RT (200 units/µl, Gibco BRL). This was incubated at 37°C for 1.5 hr, heated to 80°C for 10 min, and cooled to 4°C. The final volume yield of cDNA was approximately 25 µl. For a reverse transcription negative control (RT−), all the above reagents were added to another PCR tube, except that 2 µl of sterile water was substituted for the 100 blastocysts.

PCR was then performed to detect cDNA made from Ar mRNA during the reverse transcription step. Primer pair no. 1 or no. 2 was used and in a thin-walled PCR tube the following were placed: 38 µl sterile water, 5 µl 10× Taq DNA polymerase buffer ($MgCl_2$ free, Promega), 3 µl 25 mM $MgCl_2$ (Promega), 2 µl dNTPs (10 mM each, Promega), 0.5 µl forward primer (0.1 µg/µl), 0.5 µl reverse primer (0.1 µg/λ), and 1 µl cDNA. For a PCR negative control (PCR−), all the above reagents were added to another PCR tube except that 1 µl of sterile water was substituted for cDNA.

Samples were heated to 95°C for 4 min, at which time 1.0 µl of Taq DNA polymerase was added for a "hot start" PCR. The samples were heated for 1 additional min at 95°C. Samples were then brought to the annealing temperature of 59°C for 30 sec and to an elongation temperature of 72°C for another 30 sec. The samples were then cycled 36 times through 93°C for 1 min, 59°C for 30 sec, and 72°C for 30 sec. The last cycle was held at 72°C for 10 min rather than 30 sec. The total number of PCR cycles was 37. PCR products were visualized on an ethidium bromide-stained 2% agarose gel under UV light.

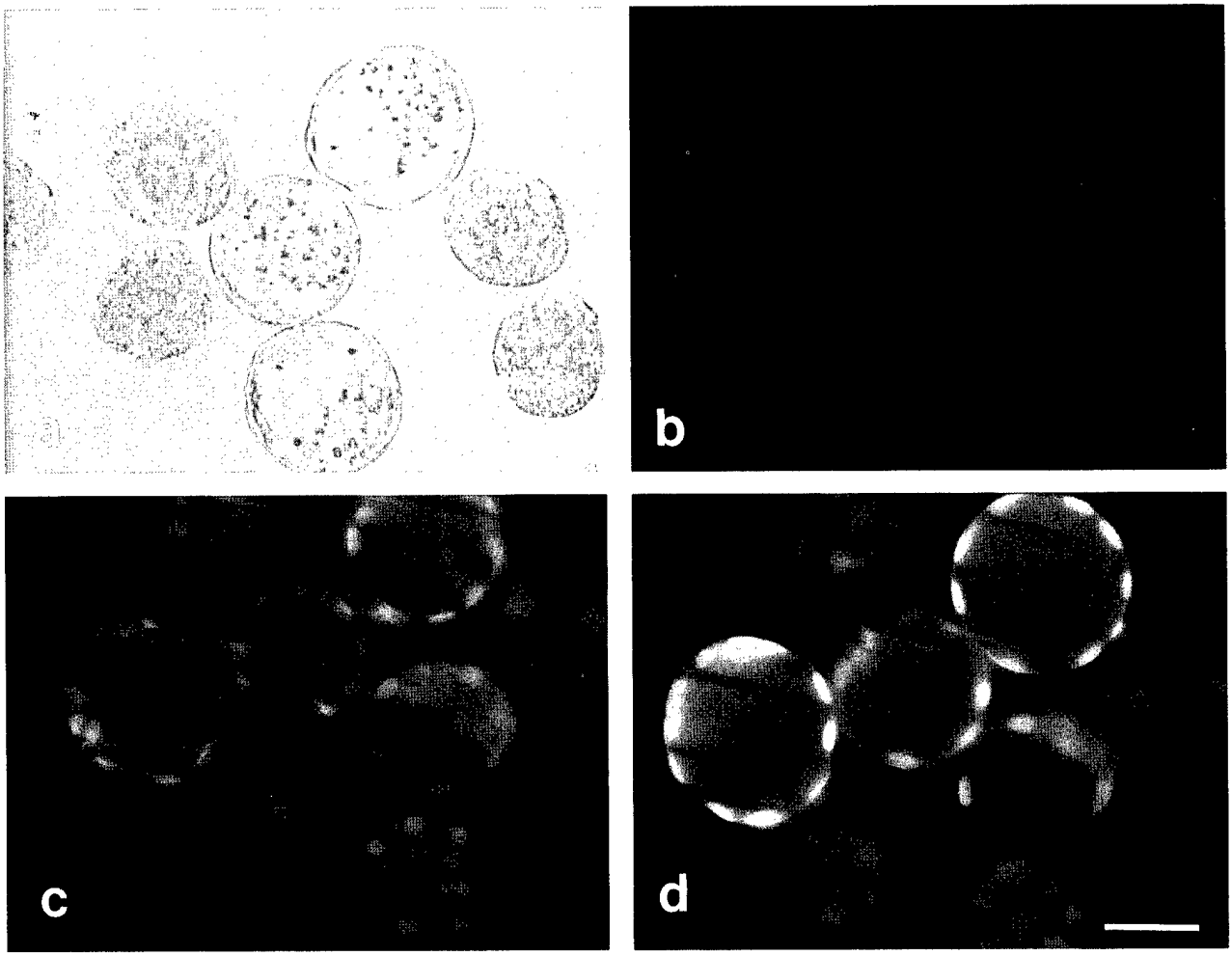

Fig. 1. Indirect immunofluorescence assays using an anti-Ar IgG showing the expression of Ar protein in morulae- and blastocyst-stage preimplantation mouse embryos. **a:** Phase-contrast image of control embryos exposed to the anti-Ar IgG that was preabsorbed with the immunizing peptide. **b:** Epifluorescence image of control embryos from panel a. **c:** Epifluorescence image of morulae- and blastocyst-stage embryos exposed to the anti-Ar IgG (40 μg/mL). Note that the rhodamine signal localizes to both the cytoplasm and nucleus. **d:** Another epifluorescence image of the same embryos from panel c with a different focus emphasizing the nuclear localization in the blastocysts. The bar in panel d represents 100 μm.

Restriction Enzyme Digests of RT-PCR Products

Primer pair no. 2 was used to generate a 613-bp product, which was then subjected to restriction enzyme digestion. EcoRI was used as a negative control since the PCR product does not contain any EcoRI recognition sites. TaqI should cut the 613 bp product only once, producing restriction fragments 432 bp and 181 bp in length. Forty units of enzyme were used to digest 10 μl of PCR product in a 25 μl volume. Samples were incubated at 37°C overnight and heat killed the following morning. Products were visualized on an ethidium bromide-stained 2% agarose gel under UV light.

Antisense Ar Oligodeoxynucleotides

Phosphorothioate-modified antisense Ar oligodeoxynucleotides (S-oligos) were prepared as highly purified products by National Biosciences, Inc. Both the antisense and control S-oligo were capped with 4 phosphorothioate linkages at the 5′ and 3′ ends. The chimeric nature of these oligos have been shown to protect them against degradation by nucleases. The sequence of the antisense S-oligo was 5′-ACCGTTCACCAAAGTAATCT-3′ which hybridizes to nucleotides 512 to 532 of murine Ar mRNA. The antisense S-oligo used is 100% similar to murine Ar mRNA and only 60% similar to other murine cDNA sequences reported in GenBank (Genetics Computer Group, 1992). The sequence of the antisense Ar S-oligo was randomized to generate a control S-oligo. The sequence of the control S-oligo was 5′-TATATATATAAAGGCCCCCC-3′ and is less than 65% similar to other known murine cDNA sequences reported in GenBank.

Embryo Culture in Antisense Ar S-Oligos

Twenty to twenty-five 4-cell embryos were placed in 15 μl drops of modified T6 culture medium that con-

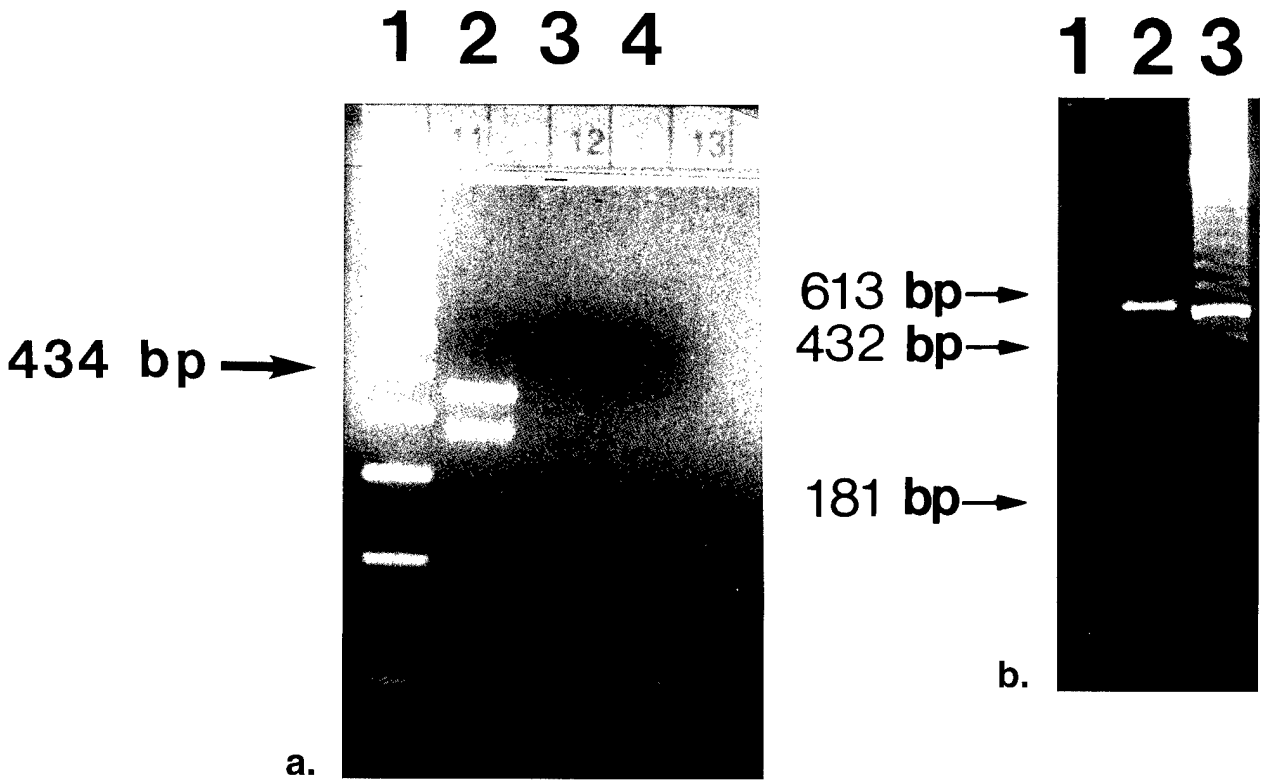

Fig. 2. a: Ethidium bromide-stained agarose gel showing the expression of Ar mRNA by mouse blastocysts using RT-PCR and primers specific for mouse Ar. *Lane 1:* 100 base pair ladder. *Lane 2:* 434 bp RT-PCR product from 100 mouse blastocysts. *Lane 3:* PCR negative. For this control, sterile water was substituted for cDNA and the sample was then subjected to PCR. *Lane 4:* RT negative. For this control, sterile water was substituted for blastocysts and the sample was then subjected to RT-PCR. **b:** Ethidium bromide–stained agarose gel showing the results of restriction enzyme digestion of the 613 bp RT-PCR product from blastocysts. The lower primer was the same as that used for Figure 2a, but a different upper primer was used which resulted in a bigger PCR product. *Lane 1:* Ar RT-PCR product treated with Taq1, an enzyme that should cut the Ar RT-PCR product once. Note the 613 bp product is cut into 432 bp and 181 bp restriction fragments. *Lane 2:* Ar RT-PCR product treated with EcoR1, an enzyme that should not cut the Ar RT-PCR product. Note that the 613 bp product is not affected. *Lane 3:* 100 bp ladder.

tained either the antisense Ar S-oligo or the control S-oligo and was overlaid with paraffin oil. Concentrations of S-oligo were 25 and 50 μM. Additional groups of 20–25 embryos were placed into 15 μL drops of culture medium lacking either the antisense or control S-oligo. The embryos were returned to the incubator and cultured for a total of 48 hr. After the first 24 hr, the embryos were transferred to new 15 μl drops of culture media containing fresh oligos and cultured for an additional 24 hr. At the end of the 48-hr culture period, the embryos were scored for incidence of blastocyst formation and fixed to obtain mean embryo cell numbers using the method of Tarkowski (1966). The embryos were fixed in a mixture of glacial acetic acid and ethanol, deposited singly onto glass slides, and blown flat. This treatment produces a spread of nuclei that are subsequently stained with Geisma to visualize nuclei, which can be easily counted under a dissection microscope.

Specificity Test for Antisense Ar S-Oligos

Because isolated Ar was unavailable at the time of these experiments, TGF-α was used in an attempt to rescue embryos from the effects of the antisense Ar S-oligo. Four-cell embryos were cultured with or without TGF-α (10 ng/ml; Sigma) in 15 μl drops of culture media containing the antisense Ar S-oligo. Control embryos were cultured in 15 μl drops of culture media containing the random control S-oligo. The S-oligos were used at a concentration of 50 μM, which would be a more rigorous test of the ability of TGF-α to rescue embryos from the effects of the antisense Ar S-oligo. If the effects were due entirely to toxicity associated with the S-oligo, then these effects should not be altered by TGF-α. After a 48-hr culture period, embryos were scored for onset of cavitation and fixed to obtain embryo cell numbers.

The ability of the antisense S-oligo to inhibit Ar protein production was also assessed using indirect immunofluorescence assays. However, these tests did not show a detectable difference in Ar protein expression between embryos cultured in the antisense S-oligo and embryos cultured in the control S-oligo. This failure may have resulted from a truncated, functionally defective Ar protein that retained its antigenic properties and thus was still recognized by the anti-Ar IgG. This possibility is consistent with the fact that this particular antisense S-oligo hybridizes to the Ar mRNA

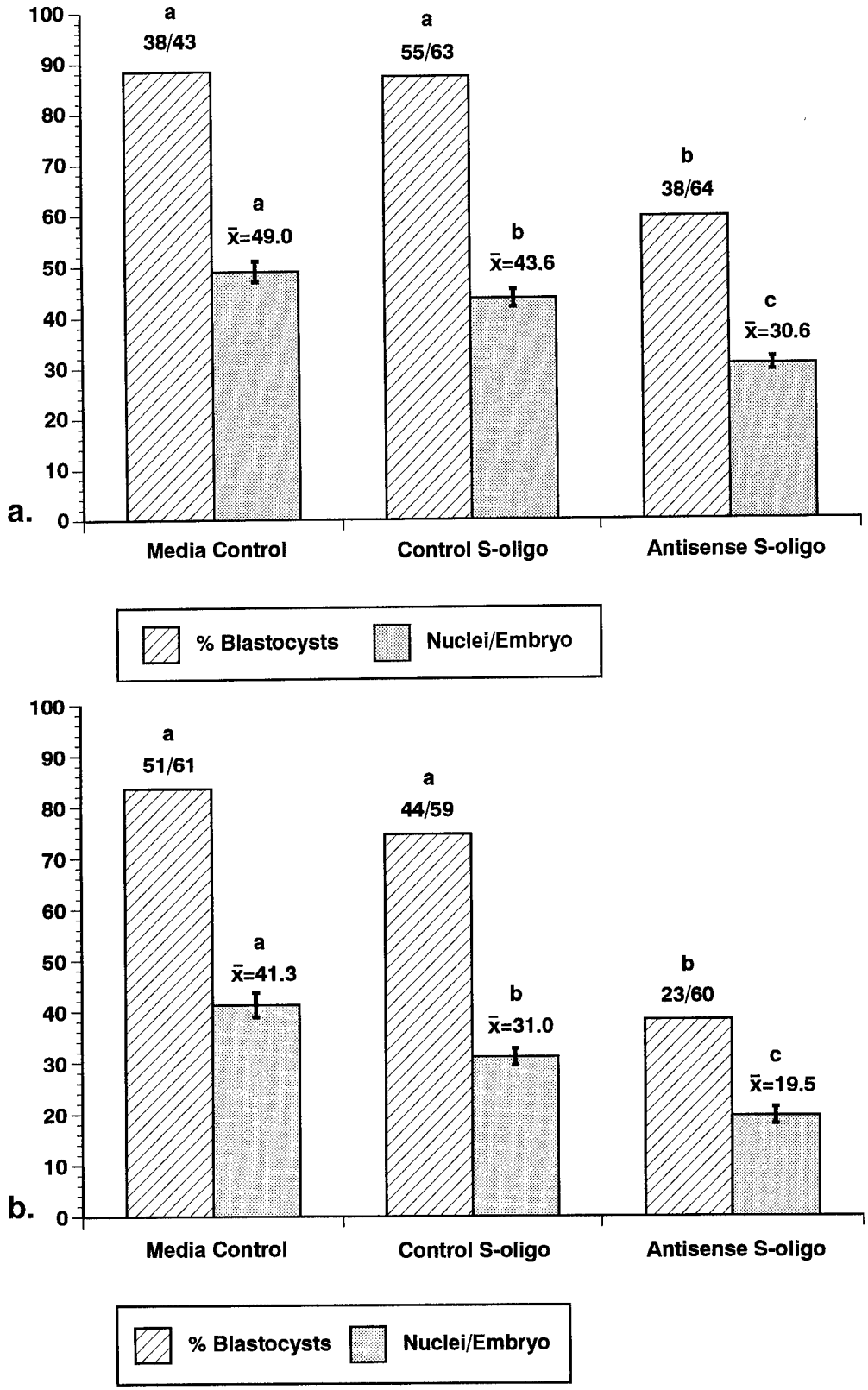

Fig. 3. a: Effect of the antisense Ar S-oligo on embryo blastocyst formation and cell number at a concentration of 25 μM. The data represented in this graph was pooled from 3 separate experiments. The percentage of embryos undergoing blastocyst formation for each group is indicated by the cross-hatched bars, and the numbers used to calculate this percentage are shown at the top of each bar. Embryos cultured in the antisense S-oligo exhibited a significantly reduced number of embryos undergoing blastocyst formation compared to controls (the letter "b" represents $P < .001$). The mean embryo cell number for each group is given at the top of each stippled bar (the letter "b" in this case represents $P < .05$, while the letter "c" represents $P < .001$). **b:** Effect of the antisense Ar S-oligo on embryo blastocyst formation and cell number at a concentration of 50 μM. The data represented in this graph was pooled from 3 separate experiments. Both "b" and "c" represent $P < .001$.

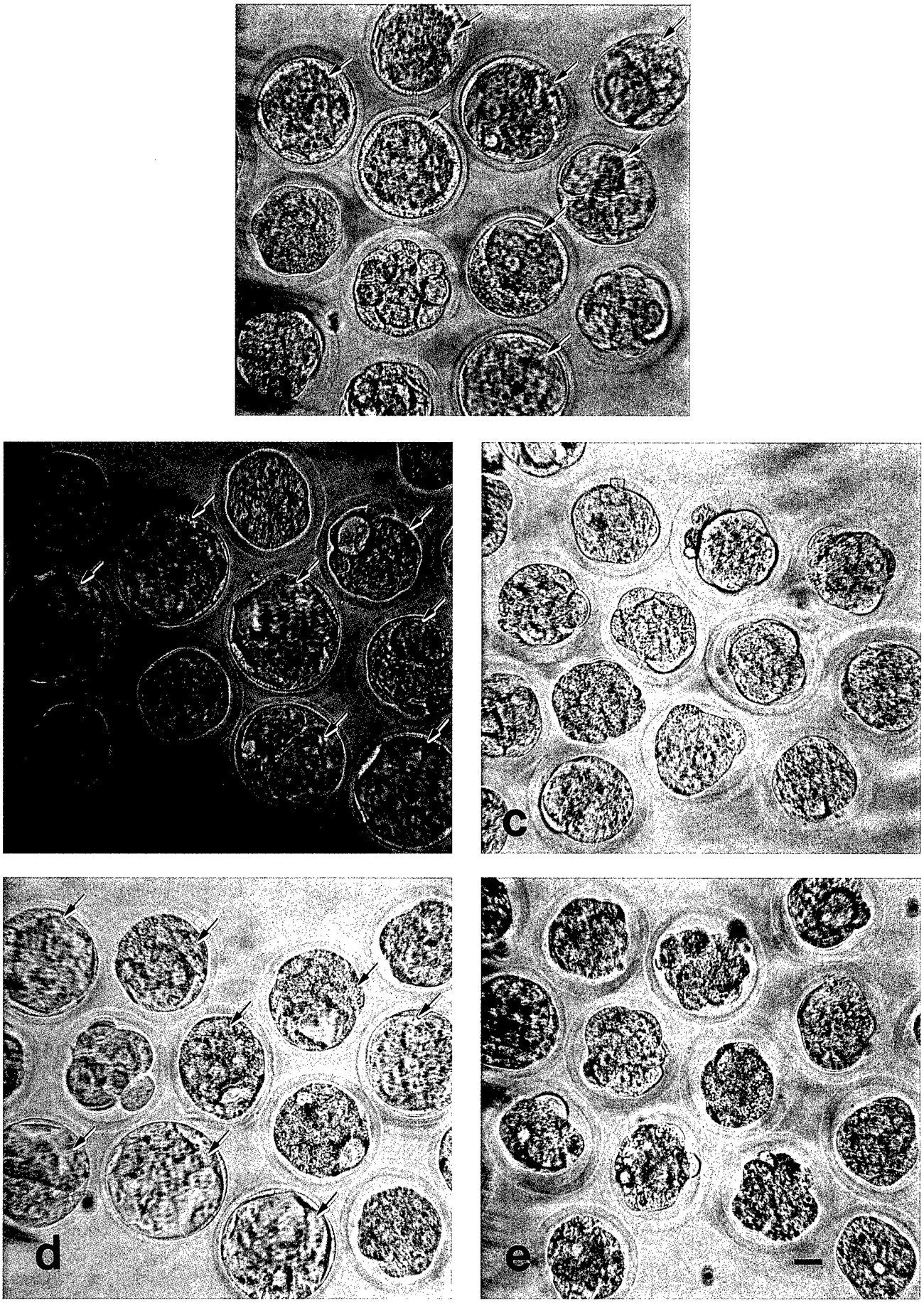

Fig. 4. Effect of the antisense Ar S-oligo on embryo morphology. Blastocysts are indicated by arrows. **a:** Embryos cultured in media without S-oligos. **b:** Embryos cultured in the random control S-oligo at 25 μM. **c:** Embryos cultured in the antisense S-oligo at 25 μM. **d:** Embryos cultured in the random control S-oligo at 50 μM. **e:** Embryos cultured in the antisense S-oligo at 50 μM. Note that the embryos in both antisense groups are delayed at the morula stage of development. The bar in panel e represents 100 μm.

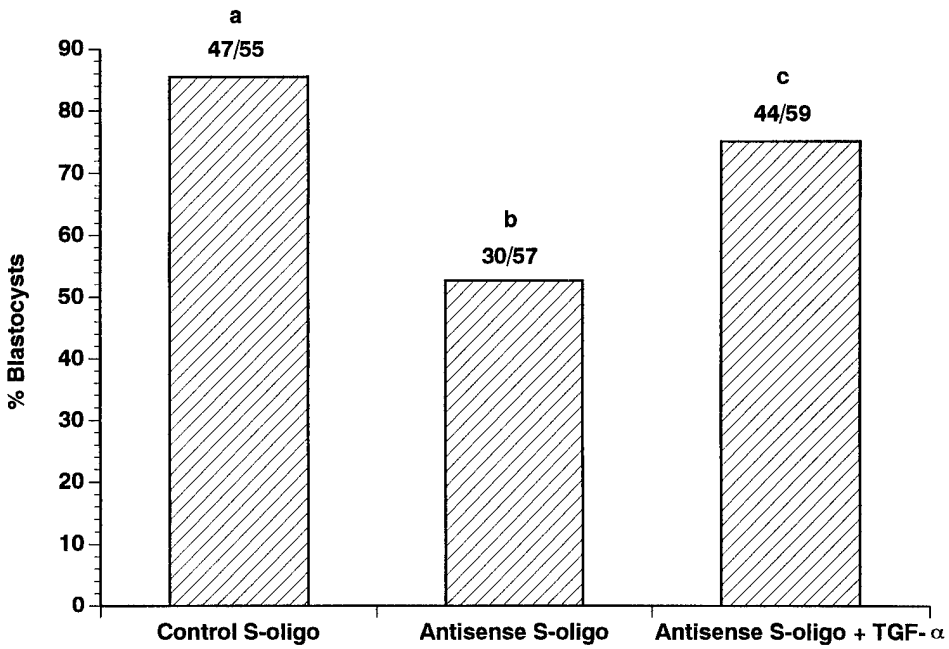

Fig. 5. Effect of culturing embryos simultaneously in the presence of the antisense Ar S-oligo and TGF-α. The data represented in this graph was pooled from three separate experiments. The embryos were cultured for 48 h and scored for incidence of blastocyst formation. The antisense S-oligo was used at a concentration of 50 μM and TGF-α was used at a concentration of 10 ng/mL. The letter "b" represents $P < .001$. The letter "c" represents $P < .001$ when compared to "b" and $P < .05$ when compared to "a."

at a position downstream of the nucleotide sequence that corresponds to the peptide used for immunization. Additionally, the precursor fragment of the Ar protein might be stabilized by its interaction with DNA.

Embryo Culture in Recombinant Amphiregulin

The 98-amino-acid-long form of recombinant Ar was purchased from R&D Systems. A stock solution was prepared by adding 1.0 ml of sterile PBS containing 2 mg of bovine serum albumin to 100 μg of lyophilized Ar. The Ar stock solutions were stored at −70°C. A sterile solution of 2 mg/ml BSA without Ar was also prepared as a control.

Approximately twenty 2-cell embryos were cultured in 20 μl drops of T6 culture medium containing Ar at concentrations of 1.0 ng/ml, 0.1 ng/ml, or 0.01 ng/ml. Control embryos were cultured in 20 μl drops of T6 media containing a matched amount of BSA. After 24 hr in culture, embryos were placed into drops of culture media containing fresh Ar and returned to the incubator. Embryos were carefully monitored for the first signs of cavitation and scored for cavitation at 2-hr time intervals. Embryos were fixed for cell numbers when at least 50% of control embryos had cavitated.

Eight-cell embryos were also cultured in the presence of Ar at concentrations of 10 ng/ml or 1.0 ng/ml. Control embryos were cultured in T6 medium containing a matched amount of BSA. Again, embryos were monitored for the first signs of cavitation and scored for cavitation at 2-hr time intervals. Embryos were fixed for cell numbers when at least 60% of control embryos had cavitated.

Statistical Analyses

Differences in mean embryo cell number were evaluated by unpaired one-tailed t-tests with a 5% significance level. Differences in percent of blastocysts were compared using chi square analyses with a 5% significance level.

RESULTS

Localization of Ar Protein by Indirect Immunofluorescence

Control embryos exposed to the anti-Ar IgG which had first been preabsorbed with the peptide antigen did not exhibit any labeling, thus confirming the specificity of the antibody (Fig. 1a and 1b). However, morula- and blastocyst-stage embryos exposed to the anti-Ar antibody exhibited both a cytoplasmic and nuclear localization of fluorescence (Fig. 1c and 1d). Figure 1c focuses on the nuclear signal produced in the morula and Figure 1d focuses on the nuclear signal produced in the blastocysts. A nuclear distribution of Ar in preimplantation embryos is consistent with the nuclear localization of Ar reported for many other cell types (Johnson et al., 1991; Johnson et al., 1992; Ebert et al., 1994; Normanno et al., 1994; Akagi et al., 1995).

RT-PCR and Enzyme Digests

To confirm the results obtained from the indirect immunofluorescence assays, RT-PCR was performed to determine if preimplantation embryos expressed the mRNA for Ar. Using primer pair no. 1, the predicted 434 bp product was detected in blastocysts (Fig. 2a, lane 2)

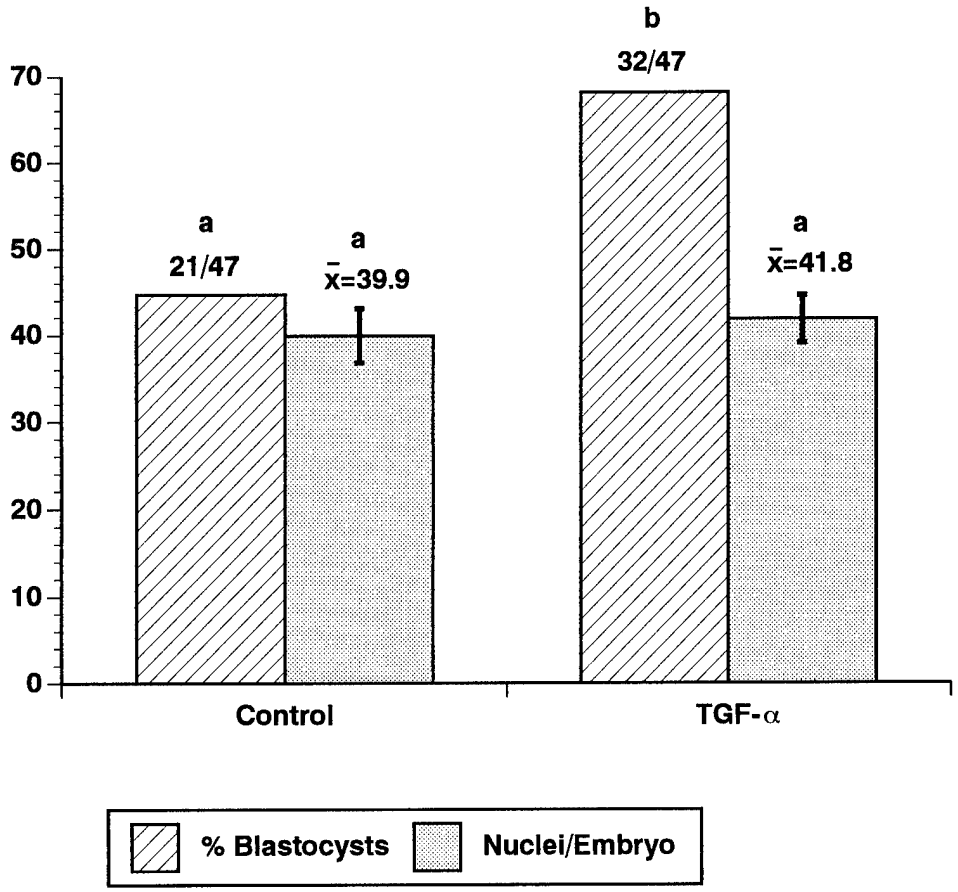

Fig. 6. Effect of TGF-α on embryo blastocyst formation and cell number. The data represented in this graph was pooled from three separate experiments. TGF-α was used at a concentration of 10 ng/mL. The letter "b" represents $P < .005$.

whereas a band was not detected in the lanes corresponding to the PCR and RT negative controls (Fig. 2a, lanes 3 and 4).

To verify the RT-PCR product was amplified from cDNA reverse-transcribed from Ar mRNA, restriction endonucleases were used (Fig. 2b). Using primer pair no. 2, the predicted 613 bp product was detected in blastocysts. When the Ar RT-PCR product was treated with EcoRI, an enzyme that should not cut the RT-PCR product, the 613 bp product remained intact (Fig. 2b, lane 2). When the Ar RT-PCR product was treated with TaqI, the 613 bp product was cut into the expected 432 and 181 bp restriction fragments (Fig. 2b, lane 1).

Embryo Culture in Antisense Ar S-Oligos

After 48 hr of culture, embryos in the antisense Ar S-oligo lagged behind embryos cultured in the control S-oligo with respect to blastocyst formation and with respect to embryo cell numbers for concentrations of S-oligo, 25 and 50 μM (Fig. 3a and 3b; $P < .001$). In addition, embryos cultured in the control S-oligo at 25 and 50 μM concentrations exhibited significantly reduced mean embryo cell numbers compared to embryos cultured in T6 media lacking S-oligos (Fig. 3a and 3b; $P < .05$ for 25 μM and $P < .001$ for 50 μM). This served as an indicator of the degree of toxicity associated with the S-oligos used in this study. Since the antisense Ar S-oligo significantly reduced both the rate of blastocyst formation and mean embryo cell number compared to embryos cultured in the control S-oligo, this suggests that some of the effects produced by the antisense S-oligo may have resulted from a sequence-specific effect of the antisense S-oligo on Ar protein function rather than toxicity associated with the antisense S-oligo.

The morphology of the embryos cultured in the antisense Ar S-oligo was very different from the morphology of embryos cultured in either the control S-oligo or T6 media alone (Fig. 4). We consistently observed timepoints when 60–70% of embryos in control groups had formed blastocoeles while 100% of the embryos cultured in the antisense Ar S-oligo were developmentally delayed at the morula stage (Fig. 4).

Specificity Test for Ar S-Oligos

TGF-α significantly improved the rate of blastocyst formation in embryos cultured in the antisense Ar S-oligo compared to embryos cultured in the antisense

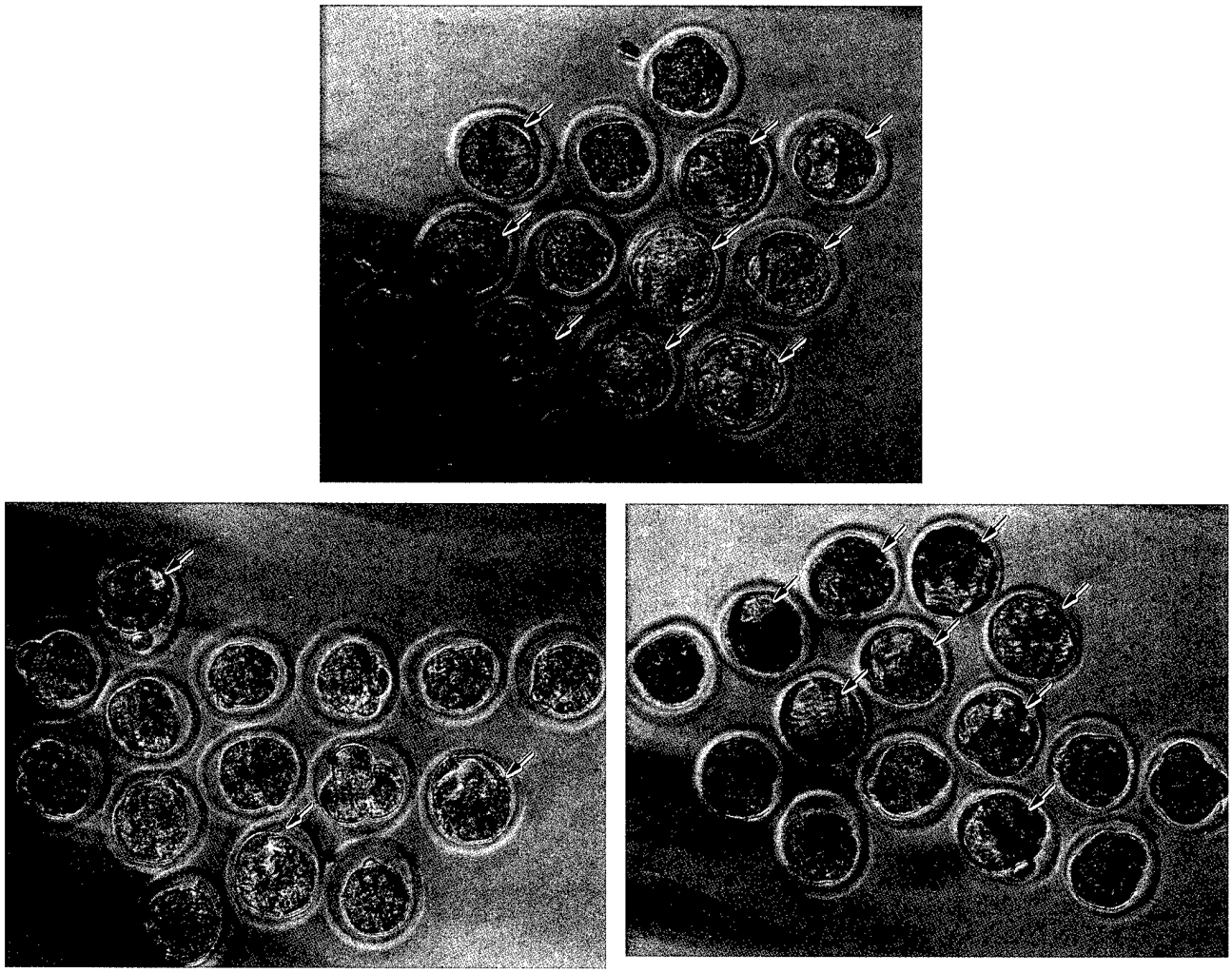

Fig. 7. Morphology of embryos cultured simultaneously in the presence of the antisense Ar S-oligo and TGF-α. Blastocysts are indicated by arrows. **a:** Embryos cultured in the random, control S-oligo. **b:** Embryos cultured in the antisense S-oligo without TGF-α. **c:** Embryos cultured in the antisense S-oligo and TGF-α. The bar in panel c represents 100 μm.

Ar S-oligo without TGF-α (Fig. 5; $P < .001$). However, the rate of blastocyst formation for these embryos was still significantly slower ($P < .05$) than for embryos cultured in the control S-oligo. In addition, embryo cell numbers were not significantly improved in embryos cultured in the antisense Ar S-oligo and TGF-α (data not shown). This suggests that TGF-α cannot compensate completely for Ar function. The inability of TGF-α to rescue embryo cell numbers was not surprising since we have found that addition of TGF-α to the culture medium of preimplantation embryos stimulates primarily blastocyst formation rather than embryo cell number (Fig. 6). The morphology of the embryos cultured in the antisense Ar S-oligo and TGF-α is shown in Figure 7.

Embryo Culture in Amphiregulin

Two-cell embryos cultured in the presence of Ar at 1.0, 0.1, and 0.01 ng/ml developed into blastocysts significantly faster than control embryos (Fig. 8a). In addition, the mean embryo cell number for embryos cultured in Ar at 0.01 ng/ml was significantly greater than the mean embryo cell number for control embryos (Fig. 8b, $P < 0.02$). Embryos cultured in Ar at 1.0 and 0.1 ng/ml also had greater mean embryo cell numbers compared to controls, but this increase was not significant.

Eight-cell embryos cultured in Ar at 10 and 1.0 ng/ml also developed into blastocysts more quickly than did control embryos (Fig. 9a, 9b). Both concentrations of Ar produced significantly greater mean embryo cell numbers compared to controls (Fig. 9c).

DISCUSSION

Here we provide the first evidence that murine preimplantation embryos express the EGF receptor ligand amphiregulin (Ar) in addition to TGF-α (Rappolee et al., 1988; Werb, 1990). RT-PCR was used to show expression of Ar mRNA in mouse blastocysts and

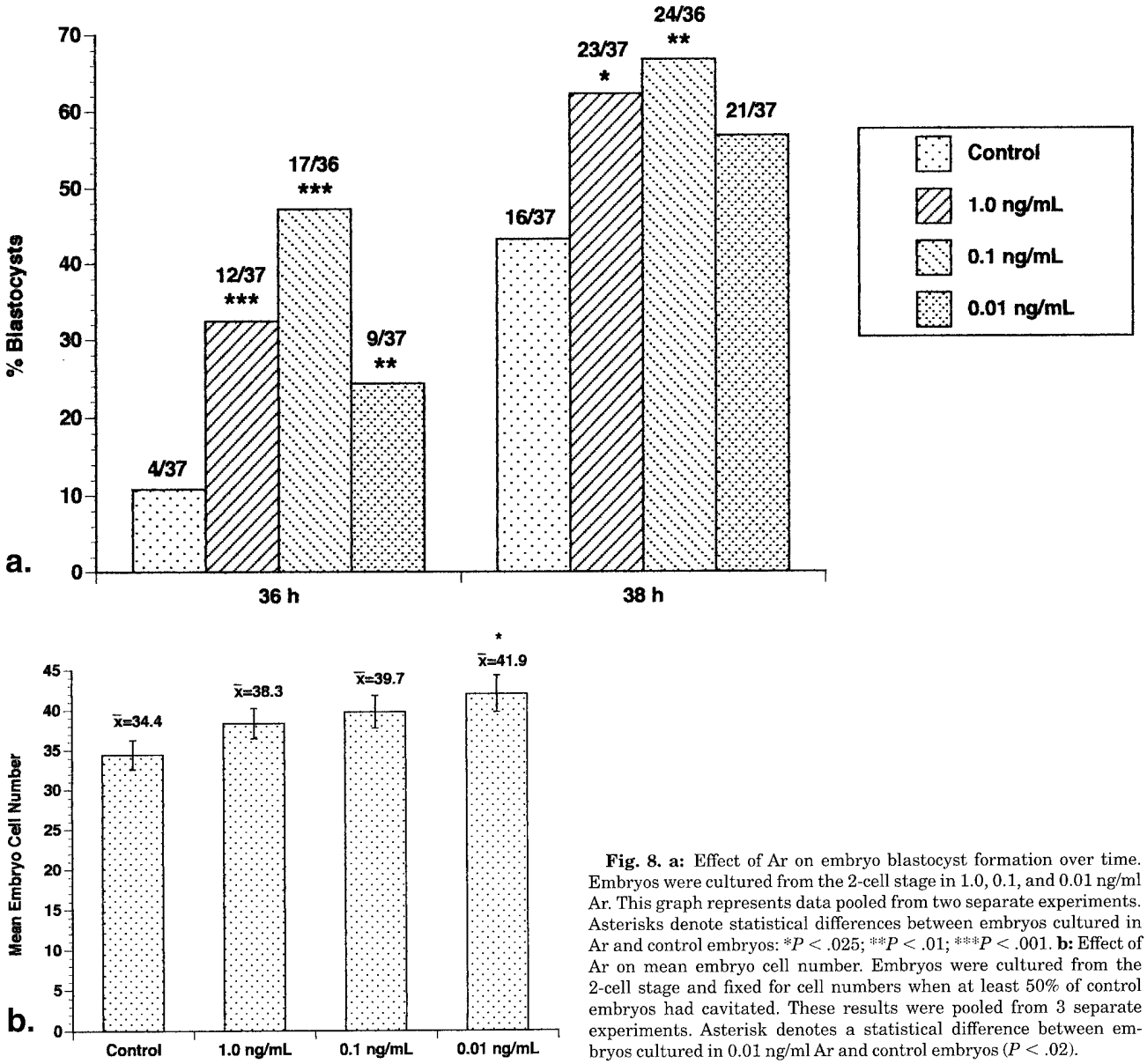

Fig. 8. a: Effect of Ar on embryo blastocyst formation over time. Embryos were cultured from the 2-cell stage in 1.0, 0.1, and 0.01 ng/ml Ar. This graph represents data pooled from two separate experiments. Asterisks denote statistical differences between embryos cultured in Ar and control embryos: $^{*}P < .025$; $^{**}P < .01$; $^{***}P < .001$. **b:** Effect of Ar on mean embryo cell number. Embryos were cultured from the 2-cell stage and fixed for cell numbers when at least 50% of control embryos had cavitated. These results were pooled from 3 separate experiments. Asterisk denotes a statistical difference between embryos cultured in 0.01 ng/ml Ar and control embryos ($P < .02$).

an affinity-purified anti-Ar IgG was used to demonstrate a cytoplasmic and nuclear localization of Ar protein in morula- and blastocyst-stge embryos. Immunohistochemical analyses of several cell lines have shown Ar to be present in both the cytoplasm and nucleus (Shoyab et al., 1989; Johnson et al., 1991; Johnson et al., 1992; Ebert et al., 1994; Normanno et al., 1994; Akagi et al., 1995). It is not clear why a previous study based on cDNA library screens failed to reveal Ar expression in mouse preimplantation embryos (Johnson et al., 1994). It is possible that the levels of Ar mRNA present in preimplantation embryos are below the detection limit of these cDNA libraries, although they were successfully screened for mRNA-encoding TGF-α (Johnson et al., 1994).

Ar was so named because of its dual roles in modulating growth: it can promote or inhibit the growth of both normal and malignant epithelial cells, depending on the concentration of Ar (Shoyab et al., 1988; Johnson et al., 1991). It is now clear that the earlier studies used a carboxy terminal-truncated form of Ar that is less active. The earlier studies should probably be repeated using the extended, more active form of Ar, like that used in the present study. Phosphorothioate-modified antisense Ar oligodeoxynucleotides reduce cell growth in a gastric carcinoma cell line and human mammary epithelial cells, suggesting that Ar acts as an autocrine growth factor for these cells (Normanno et al., 1994; Akagi et al., 1995). This negative effect on cell proliferation is similar to what we observed for embryos cultured in antisense Ar S-oligos (Fig. 3a, 3b). In the work presented here, we used an antisense Ar S-oligo that should prevent the synthesis of sequences corresponding to the third disulfide loop in mature Ar and the

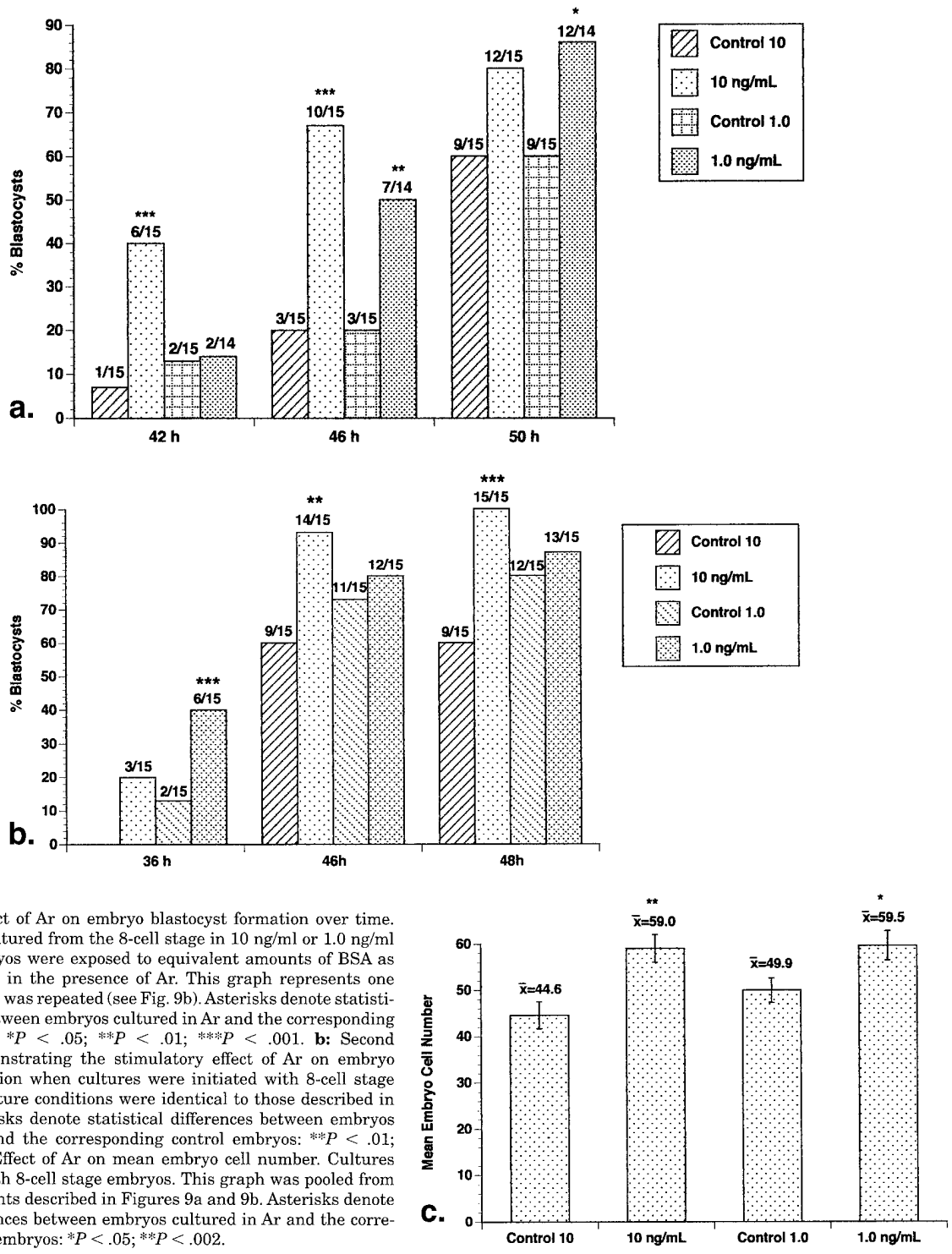

Fig. 9. a: Effect of Ar on embryo blastocyst formation over time. Embryos were cultured from the 8-cell stage in 10 ng/ml or 1.0 ng/ml Ar. Control embryos were exposed to equivalent amounts of BSA as embryos cultured in the presence of Ar. This graph represents one experiment which was repeated (see Fig. 9b). Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $*P < .05$; $**P < .01$; $***P < .001$. **b:** Second experiment demonstrating the stimulatory effect of Ar on embryo blastocyst formation when cultures were initiated with 8-cell stage embryos. The culture conditions were identical to those described in Figure 9a. Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $**P < .01$; $***P < .005$. **c:** Effect of Ar on mean embryo cell number. Cultures were initiated with 8-cell stage embryos. This graph was pooled from the two experiments described in Figures 9a and 9b. Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $*P < .05$; $**P < .002$.

transmembrane and cytoplasmic domains contained in the precursor. The 6 cysteines and their disulfide loops in EGF and TGF-α are essential for biological activity (Heath et al., 1986). The transmembrane domain would be required for insertion of the Ar precursor into the plasma membrane to provide the potential of cell membrane-bound Ar interacting with EGF receptors on adjacent cells. Stimulation of EGF receptors by membrane-anchored TGF-α is known to occur and has been termed juxtacrine stimulation (Brachmann et al., 1989; Wong et al., 1989; Anklesaria et al., 1990). In our experiments, as well as those using other cell types, it is possible for the antisense Ar S-oligo to have impaired production of both soluble and membrane-bound forms

of Ar—if either or both forms are indeed synthesized by embryos and these other cell types. At this time there is no published information regarding the potential for an interaction between cell membrane-bound Ar and EGF receptors on adjacent cells.

The antisense Ar S-oligo significantly reduced mean embryo cell number and reduced the rate of blastocyst formation when compared to control embryos cultured in a random control S-oligo at 50 and 25 μM. Culturing embryos in the presence of both TGF-α and the antisense Ar S-oligo reversed the antisense effect and significantly improved the rate of blastocyst formation, but not mean embryo cell number. These observations indicate that TGF-α may substitute partly, but not entirely, for Ar function. The inability of TGF-α to rescue cell numbers for embryos cultured in the antisense Ar S-oligo might be due to the unique ability of Ar to localize to the nucleus and bind to DNA. Support for this hypothesis comes from the observation that nuclear localization of schwannoma-derived growth factor (SDGF), the rat homologue of Ar, is essential for its mitogenic activity (Kimura, 1993).

The capacity of added TGF-α to offset the effects of the antisense Ar S-oligo on blastocyst formation served as a specificity control for this particular S-oligo. The rescue of blastocyst development by TGF-α in this experiment suggests that the antisense Ar S-oligo may have specifically affected Ar protein function. The antisense Ar S-oligo was used at a concentration of 50 μM as a stringent test for specificity. If the effects on blastocyst formation produced by the antisense Ar S-oligo at 50 μM were due entirely to toxicity, then one would not expect TGF-α to have had any effect on blastocyst formation. However, improved blastocyst development was observed.

Recombinant Ar has recently become commercially available, and we therefore tested the hypothesis that Ar added to the culture medium of preimplantation embryos might stimulate blastocyst formation and embryo cell numbers, as predicted by our antisense Ar studies. Indeed, preimplantation embryos cultured in Ar from both the 2-cell and 8-cell stages exhibited enhanced blastocyst development and increased mean embryo cell numbers compared to controls. As stated earlier, we have found that addition of TGF-α to the culture medium of preimplantation embryos stimulates primarily blastocyst formation rather than embryo cell number. Other studies have reported similar findings. For example, TGF-α has been shown to stimulate blastocoel expansion but not mean embryo cell number (Dardik and Schultz, 1991). Paria and Dey (1990) found that EGF did not increase embryo cell number when embryos were cultured in groups or singly in 50 μL drops of culture medium; EGF and TGF-α were observed to stimulate both blastocyst formation and embryo cell number when embryos were cultured singly in drops of 25 μL. Although Paria and Dey reported that EGF had no effect on blastocyst formation when embryos were cultured in groups, our studies show a clear stimulation of blastocyst formation by both TGF-α and by Ar. This apparent discrepancy may be due to how the data was collected. In our studies, we routinely monitor embryos for the first signs of cavitation and at 2-hr intervals thereafter. As a result, we can be sure to record any differences in the *rate* of blatocyst formation as well as in the final incidence of blastocyst formation between control embryos and those cultured in the growth factor. After a total of 48 hr in culture, similar numbers of blastocysts are often produced in each group. Paria and Dey state that no differences in development were observed between control embryos and those cultured in EGF after 48 hr in culture. It is possible, however, that differences in the progression of blastocyst formation could have occurred, prior to the 48-hr timepoint, as we observed in our studies.

In summary, we have provided evidence that murine preimplantation embryos express the mRNA and protein for an additional EGF receptor ligand, Ar. We further propose that Ar shares with TGF-α the functions of promoting blastocyst formation and cell proliferation by initiating signal transduction cascades that serve activated EGF receptor. However, our results suggest that Ar may be more mitogenic in preimplantation embryos than is TGF-α. We speculate that this difference may be due to the unique capacity of Ar to localize to the nucleus.

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Cell Death and Differentiation

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

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Running Title: Cripto in mammary cell growth and survival

Abstract

The expression of Cripto, a member of the EGF-like family of ligands, is highly correlated with transformation in breast cancer. Eighty-two percent of breast carcinomas express Cripto whereas it is undetected in normal human breast tissue. We confirmed and extended findings that Cripto 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. Cripto 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 Cripto 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 overexpressing Cripto did not differentiate efficiently. Infection of CID 9 cells with a Cripto 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 Cripto is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the inhibition of breast cell differentiation by Cripto. This indicates a possible clinical approach for mammary tumor intervention. Our results showed that Cripto stimulated the growth and survival of mammary cells, but did not stimulate tumorigenesis *in vivo*.

Keywords: Retroviruses, overexpression, antisense, milk proteins, apoptosis, tumorigenicity.

Introduction:

Cripto (CR-1) belongs to the Epidermal Growth Factor (EGF)-like family of ligands that includes transforming growth factor-alpha (TGFα), heregulin (HRG), and amphiregulin (AR). The ligands differ in tissue of origin, binding affinities and mitogenic activities, but all are candidates for autocrine, juxtacrine, or paracrine effects in vivo. This family of proteins contain "EGF-like domains" with a highly conserved structure of three disulfide loops. Cripto has the 6 conserved cysteines that make up the EGF motif but the A-loop is deleted and the B-loop is truncated. At the carboxyterminus, an additional six-cysteine motif is present and conserved also in an analogous protein recently isolated from *Xenopus laevis*. (Kinoshita *et al.*, 1995). A related mouse protein, cryptic, was recently cloned (Shen *et al.*, 1997). Cripto does not bind to EGF-receptor or to ErbB2, -3, or -4, although it binds to breast cell lines and stimulates tyrosine phosphorylation of several proteins (Aguilar-Cordova *et al.*, 1991). It has been proposed that in *Xenopus*, Cripto may interact with an FGF receptor (Kinoshita *et al.*, 1995). Although there is no hydrophobic domain in the human gene product, the mouse protein has a signal sequence and is secreted (Brandt *et al.*, 1994b) (Dono *et al.*, 1993; Normanno *et al.*, 1994). It has been proposed that the EGF-like sequences function as protein-protein contact motifs required for transfer of information.

No precise function has been attributed to Cripto but 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.*, 1994b). 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 also 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.*, 1996). 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.

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). CR-1 codes for a protein of 188 amino acid residues termed Cripto or *TDGF1* for teratocarcinoma derived growth factor-1(Ciccodicola *et al.*, 1989) . One murine gene, *Tdgf1* or Cr-1, and two intronless pseudogenes, Cr-2 and Cr-3 or *Tdgf2* and *Tdgf3*, have been isolated and characterized (Dono *et al.*, 1993; Liguori *et al.*, 1996). Mouse Cr-1 codes for a protein of 171 amino acids with 93% similarity to its human counterpart in the EGF-like domain. 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.*, 1994b).

In the present study, CID 9 cells were used to examine the expression of Cripto-1 and to determine the effects of over and underexpression 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 calf serum (FCS) and in the presence of lactogenic hormones (Schmidhauser *et al.*, 1990). We show here that Cripto is differentially expressed during mammary gland development and is expressed in a hormone dependent fashion in the normal mammary epithelial cell line. Inhibition of Cripto expression caused a change in cell morphology, decreased cell growth, increased programmed cell death and reduced anchorage independent growth. Mouse Cripto overexpression stimulated anchorage dependent and independent cell growth and decreased the differentiation potential of the mammary cells.

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 (Fig. 1, lane 2 shows cripto 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 (Fig. 1, lane 1). These results are in partial agreement with Qi et al.(Qi *et al.*, 1994) in which no Cripto expression was seen in the virgin or post-involution stage of human mammary gland development. Cripto is also expressed in CID 9 cells, derived from 14.5 day pregnant mammary glands, as a 24 kDa protein (Fig. 1, lane 7). When the cells differentiated on basement membrane substrates, they formed aggregate structures, started to express casein and higher levels of Cripto protein. The protein was also modified to Mr 26 and 28 kDa (Fig. 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 hypothesis by experimental manipulation of Cripto 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 (Fig 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 & 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. More than 250 clones were selected in G418 and pooled because the CID 9 cells are a heterogeneous cell population.

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 (Fig 3) and showed that Cripto was overexpressed at levels greater than two-fold higher than control vector populations (Fig 3). In the antisense populations only approximately one-fourth of the amount of Cripto was expressed compared to the controls (Fig 3). Thus, the retroviral vector constructs effectively modulated Cr-1 expression in CID 9 cells. Both cytokeratin positive and vimentin positive cells were observed in all the populations (data not shown). Thus, all the populations contain the two distinct cell types described by Desprez et al. (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 (Fig 4A). No differences were

observed in the morphology of cells after infection with the empty vector compared to uninfected CID 9 cells (Fig 4, panels A 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. Cripto overexpressing cells formed more frequent and larger domelike structures (Fig 4C). Cells expressing antisense Cripto were different; 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 (Fig 4D) and at confluence, they did not form domes. However, like the parental cells, they 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.

Cell proliferation and apoptosis

To determine if over and underexpression of Cripto 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 hours. Then the cells overexpressing Cripto grew faster so that at 48 and 72 hours there were a greater number of cells compared to the control. The cells underexpressing Cripto appeared to reach confluence by 24 hours and the level of cells subsequently decreased after 48 hours suggesting that they were starting to die (Fig 5A).

To determine cell densities at confluence, cells were grown in 2% FCS, allowed to grow for 5 days and remain at confluence for 2 days and then counted (Fig 5B). The Cripto 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 Cripto, the

density at confluence remained at 1.2 x 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 Cripto lowered the requirement of CID 9 cells for growth factors, cell proliferation assays were also performed by plating the cells in media containing 2% FCS 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% FCS conditions. The CID 9 cells containing the retrovirus with Cripto 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 Cripto expression might affect survival of cells even in serum-containing cultures. Apoptosis levels were assessed by nuclear morphology and by TUNEL assays. Numbers of apoptotic nuclei (Figure 6B) in log phase CID 9 cells were 0.60% ± 0.20 of the population in control pGCEN cells; this rate decreased to 0.28% ± 0.16 ($p < 0.05$) in Cripto overexpressing cells and increased to 1.53% ± 0.66 ($p= 0.035$) in antisense cells. More frequent apoptotic nuclei were observed in antisense-expressing cells (Fig. 6A, lower panel) than in either of the other two cell populations. 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-expressing 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 (Fig 7, A and B). The normal cells formed colony-like domes but Cripto underexpressing CID 9 cells on the other hand showed very little colony formation compared to control cells and ceased to proliferate after reaching confluence (Fig 7C). The CID 9 populations showed differential abilities to grow in soft agar (Fig 7). Control cells formed 15 ± 3 colonies per dish or 0.04 % of the plated cells formed colonies (Fig

7D). Whereas the Cripto overexpressing cells formed greater than 79 ± 6 colonies or 0.2% of plated cells formed colonies (Fig 7E). The antisense cells did not grow in soft agar and were therefore anchorage dependent for growth (Fig 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, 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 (Fig 8A). The CID 9 cells overexpressing Cripto grew faster than the control cells (data not shown) and therefore there were more cells, however, no mammosphere structures formed (Fig 8B). This suggests that overexpression of Cripto in cells overrides the 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 (Fig 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 a-actinin (Fig 9, lower panel). The CID 9 cells overexpressing Cripto showed only about half the amount of β-casein expression as the control CID 9 population (Fig 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 CID 9 or the 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 Cripto is not involved in β-casein expression and b) that mammospheres are not important for

differentiated expression although we cannot rule out the requirement for cell interaction. 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 (Fig 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.*, 1995) was produced by the CID 9 cells under various conditions of growth. Unlike β-casein expression, this protein was expressed in the cells whether lactogenic hormones were present or not. This protein was expressed at approximately the same level in all but one of the cell populations. The exception was the Cripto overexpressing CID 9 cells grown on matrigel in the presence of lactogenic hormones, which expressed only 15% of the level of expression of the 22 kDa protein observed in the other populations (Fig 9, lane 2). This property further distinguished the Cripto overexpressing cells.

CID 9 cells in vivo

To determine if the over or underexpression of Cripto had an effect on mammary gland development in vivo, we transferred CID 9 cells into the cleared fat pads of Balb/c mice. Uninfected CID 9 cells formed tumors in the transgenic fat pads, 5 to 6 weeks after transfer. Control mammary glands that were sham operated remained normal. Thus, normal CID 9 cells are tumorigenic when transferred to normal mammary gland tissues in vivo. 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. Western blot analysis showed that approximately one-fifth the amount of Cripto was expressed in the tumors formed from antisense Cripto 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 next tested Cripto expression effects in vivo with the parental cell line of the CID 9 cells, Comma-1D cells. Uninfected Comma-1D cells transferred to cleared mammary fat pads produced hyperplastic outgrowths at the injection site in agreement with previous studies (Aguilar-Cordova *et al.*, 1991). Comma-1D cells infected with the empty pGCEN and with the Cripto-expressing vector also formed hyperplastic outgrowths of similar sizes in numerous cases for each cell line. In contrast, Comma-1D cells underexpressing Cripto were unable to grow at all in cleared fat pads. Finally, primary cultures expressing Cripto are able to reconstitute normal mammary epithelium after transfer to cleared fat pads. In a small survey (3 mice), we found that antisense infected primary epithelial cells also would not repopulate the cleared mammary fat pad.

Taken together, these results suggest that the reduction or loss of Cripto expression renders mammary cells less able to grow and more likely to apoptose, but does not affect their ability to differentiate.

Discussion

The mammary gland is a highly complex system of interacting cell types. It contains 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 6 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 spacing of the ducts and their morphology can be experimentally modified by the origin of the mesenchyme in which they are grown (Sakakura *et al.*, 1976; Sakakura *et al.*, 1979) therefore mammary epithelial development is influenced by mesenchymal cells in the surrounding stroma. 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; Gavin and McMahon, 1992; Robinson *et al.*, 1991). Each gene is expressed differentially during these developmental stages. We show here that Cripto 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 Cripto 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 Cripto was expressed at higher levels when cells were stimulated to grow and differentiate by the presence of lactogenic hormones and extracellular matrix to lactogenic phenotypes, similar to pregnant glands in vivo. This strengthens the hypothesis that these hormones up-regulate Cripto 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 Cripto mRNA in the virgin mouse mammary gland, although no 24 or 26 kDa proteins were observed. We were able to detect low amounts of a Cripto-protein of 26 kDa in virgin glands of 8 week and older mammary glands (Fig 1, lane 1). 24 kDa is the predicted and expected size of the

mouse secreted Cripto protein, demonstrated by Brandt et al. (Brandt *et al.*, 1994a) to be authentic Cripto. In our analyses using immunoblotting of mammary gland tissues and cell lysates, we detected two Cripto proteins of 24 and 26 kDa (Fig 1) which are likely glycosylated or myristylated differently (Brandt *et al.*, 1994a). Cripto protein is expressed in undifferentiated F9 cells as a protein of 24 kDa (Fig 1, lane 5) but this not produced by differentiated F9 cells (our unpublished data). This is in agreement with previous experiments showing Cripto mRNA expression in undifferentiated but not in differentiated mouse F9 and human embryonal carcinoma cells (Ciccodicola *et al.*, 1989).CID 9 cells differentiate in vitro and we were able to determine that Cripto has an inhibitory influence on differentiation. We showed that proliferation increased and cell death decreased in CID 9 cells overexpressing Cripto while differentiation was inhibited as assessed by milk protein production (Fig 9). Two separate assays, mammosphere formation and milk protein expression, showed that overexpression of Cripto led to decreased differentiation. In agreement with our data, a 47-mer containing the EGF-like motif of human Cripto 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 Cripto stimulated the proliferation needed to bring the cells to the state required (equivalent to pregnancy) for differentiation (lactation) but inhibited the differentiation process.

Cripto appears to have a neutral or negative role in β-casein expression, and although it increases the number of domes in monolayer cultures, it inhibits the formation of mammospheres in Matrigel cultures that are thought to indicate differentiation. The antisense populations were notable in having aberrant morphologies both in differentiated and undifferentiated populations. This is not because of clonal selection because all our cell populations contain both keratin- and vimentin-positive cells (Desprez *et al.*, 1993). The antisense cells are flatter and larger when grown on plastic. The cells do not form mammospheres but do draw together to form aggregates when grown on an extracellular

matrix in the presence of lactogenic hormones. This suggests that Cripto may have an effect on cell-cell and/or cell-matrix interactions, and this possibility is currently being investigated.

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. (Marte *et al.*, 1995) in HC11 cells which were also derived from COMMA-1D cells. Milk proteins are never observed in NOG-8 mammary epithelial cells or NIH3T3 fibroblasts (Redmond *et al.*, 1988). The 22 kDa milk protein appeared to be specifically down regulated in CID 9 cells overexpressing Cripto 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 Cripto is an oncogene? We show that aberrant expression of Cripto in breast cells affected multiple characteristics of the cells. Direct analyses of proliferation and cell death rates showed that Cripto overexpression allowed growth and survival advantages. Cripto also caused the cells to become less contact inhibited and allowed them to grow in domes and also to grow anchorage independently (Fig 6). Together these results suggest that Cripto overexpression leads to a transformed phenotype in vitro. This would characterize Cripto as an oncogene. Moreover, when Cripto 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 (Fig 5). This clearly suggests that Cripto 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 Cripto 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 vivo tests do not lend support to a transforming activity of Cripto in mammary cells. CID 9 cells are an excellent model for "normal" mammary epithelial cell growth and differentiation in vitro, however, they proved to be tumorigenic when transplanted into syngenic cleared fat pads. The isolation and other procedures used to obtain CID 9 might have rendered them tumorigenic, or during the continuous passages they might have gradually become transformed. We showed here that CID 9 cells (infected with empty vector) were able to grow in soft agar, suggesting they were already transformed. COMMA-1D cells transplanted into syngeneic fat pads also gave hyperplastic growth whether they were controls or over-expressing Cripto. COMMA-1D cells infected with the antisense-Cripto retroviruses were inhibited in colony forming assays, indicating that these cells are much more sensitive to cell death than CID 9 cells in the absence of Cripto. It is informative that NOG-8 mouse mammary cells even when over-expressing Cripto do not form tumors in nude mice (Ciardiello *et al.*, 1991) suggesting that Cripto is not a transforming oncogene.

Some questions about Cripto need further study: What is the receptor for Cripto in CID 9 cells? What is the precise role of Cripto in breast carcinoma, could it act as a survival or antiapoptotic factor? The expression of Cripto is developmentally regulated in the mouse embryo (Dono *et al.*, 1993; Johnson *et al.*, 1994), but its role is unknown. Cripto first appears as a rare transcript in the blastocyst stage (day 4 postconception in mouse) in both the inner cell mass (ICM) and trophectoderm. High expression of Cr-1 in mouse and human embryonal carcinoma cells suggests that Cr-1 could play a role in teratocarcinoma formation.

In summary, the results suggest that Cripto 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 is not directly involved in differentiation. Cripto 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 Cripto's regulation and role 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 mg/ml). For differentiation, the cells were grown for 7 days in the presence of lactogenic hormones (insulin 5mg/ml, hydrocortisone 1 mg/ml, and prolactin 3mg/ml) on Matrigel (Collaborative Biomedical Products, Bedford, MA). The cells were plated in 1:1 DMEM:F12 Nutrient mixture, 2% FCS, 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 FCS 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 method described by Stoker *et al.* (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) (Fig 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 Cripto coding sequence was inserted into the XhoI site of the polylinker region in both sense and antisense orientations. PA317 producer cells (Miller and Buttimore, 1986) were transfected with 20 mg 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 Cripto either in the sense or antisense orientation or the empty vector. The cells were plated at 8 x 10^5 cells per 100 cm^2 dish and the next day fed with 8 mls of viral supernatant (prefiltered through a 0.45 mm filter), 8 mls Hams F12 media, 4 μg/ml polybrene and 5 μg/ml insulin. After 24 h the infected cells were selected using G418 (400 mg/ml) in the media. Greater than 250

colonies were pooled and used in the studies as a mixed population. Because both Cripto (sense and antisense) 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 PBS and lysed in sample buffer. Cells grown on the extracellular matrix were first treated with dispase (Collaborative Biomedical Products) for one hour 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).

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.*, 1995) 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 x 10^5 cells per fat pad in 5 to 10 ml 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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Figure legends

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 XhoI site (X). IRES, encephalomyocarditis virus internal ribosome entry site; LTR, Moloney murine leukemia virus Long Terminal Repeat sequences; neo, bacterial neomycin resistance gene.

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). a-Actinin was used to show relative protein levels in each lane. B) Graph of the percentage of Cripto expression $\pm$ SD 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%.

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 Cripto expression vector (sense), and the antisense Cripto 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 Cripto. Values are expressed as a percentage of the CID 9 cells containing the control pGCEN vector. Bars, SD.

Figure 6: Cripto's effect on cell survival. A) In situ detection of apoptotic cells with the TUNEL method (left panels) and phase contrast microscopy (right panels) of CID 9 cells expressing various levels of Cripto. All cells were plated at the same density and grown under the same conditions. Top panels are control cells containing the empty pGCEN vector. Middle panels are cells overexpressing Cripto (sense). Bottom panels are cells underexpressing Cripto (antisense). One representative field of each population is shown. B) Apoptotic levels of log phase CID 9 populations. Bars, SD.

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 Cripto (sense) show a 3-fold greater number of colonies both on plastic (B) and in soft agar (E). Whereas, CID 9 cells underexpressing Cripto (antisense) shows very little or no colony formation on plastic (C) or in soft agar (F).

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 Cripto, no mammosphere structures 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.

Figure 9: Immunoblots to show proteins produced by populations of CID 9 cells grown under various conditions. CID 9 cells underexpressing Cripto (A), overexpressing Cripto (S) or expressing normal levels of Cripto 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.

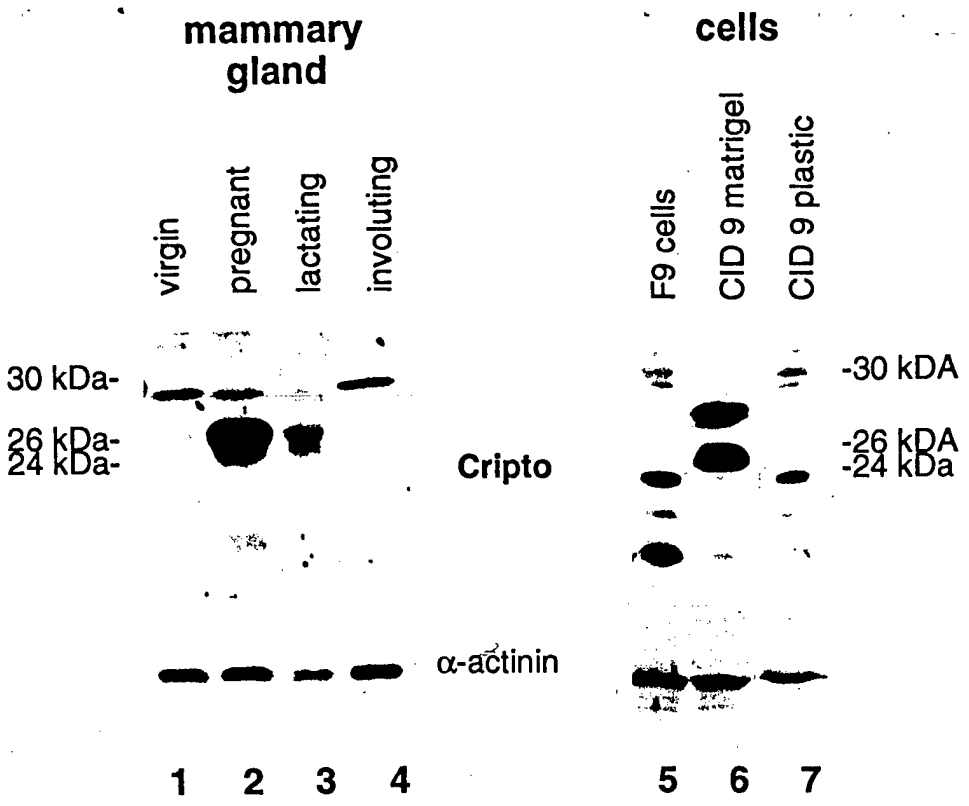

mammary gland
cells
virgin
pregnant
lactating
involuting
F9 cells
CID 9 matrigel
CID 9 plastic
30 kDa-
26 kDa-
24 kDa-
Cripto
α-actinin
-30 kDA
-26 kDA
-24 kDa
1 2 3 4 5 6 7

FIGURE 1

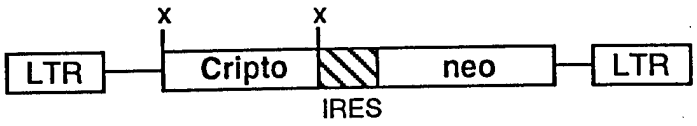
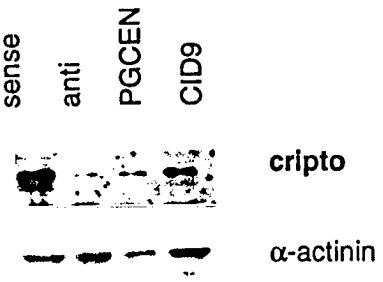


Figure 2

A.


sense
anti
PGCEN
CID9
cripto
α-actinin

B.

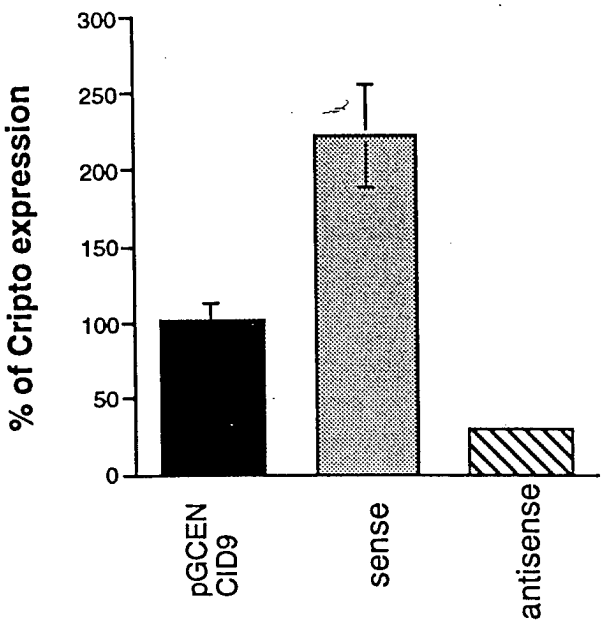

% of Cripto expression
300
250
200
150
100
50
0
pGCEN
CID9
sense
antisense

FIGURE 3

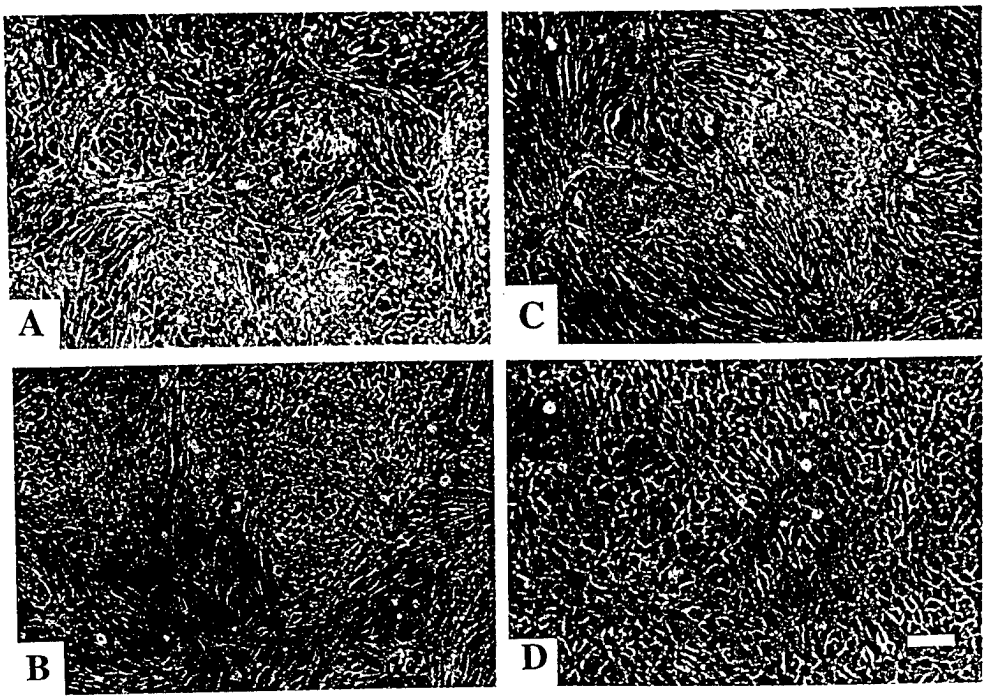


FIGURE 4

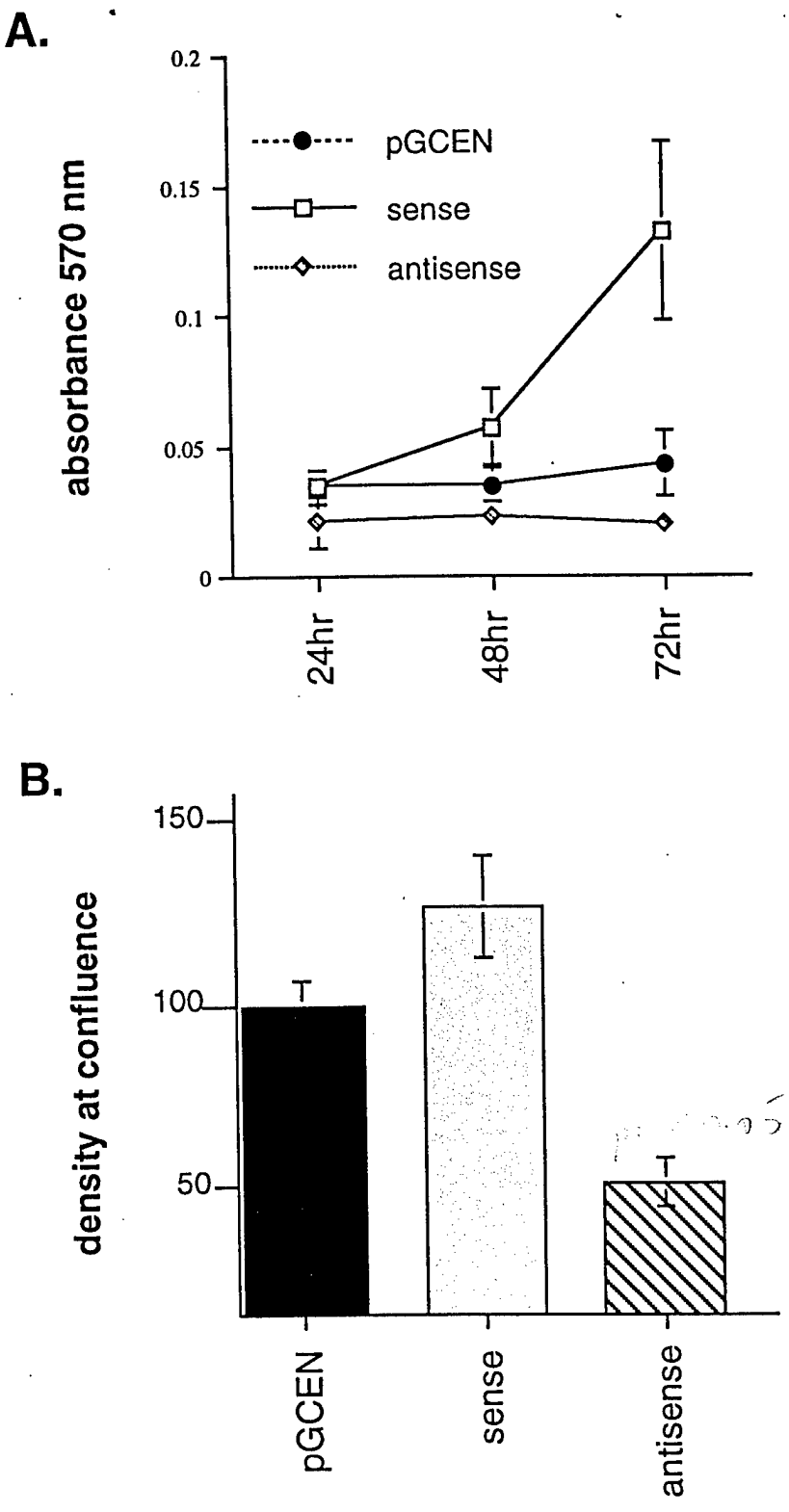

A.
absorbance 570 nm
0.2
0.15
0.1
0.05
0
pGCEN
sense
antisense
24hr
48hr
72hr
B.
density at confluence
150
100
50
pGCEN
sense
antisense

figure 5

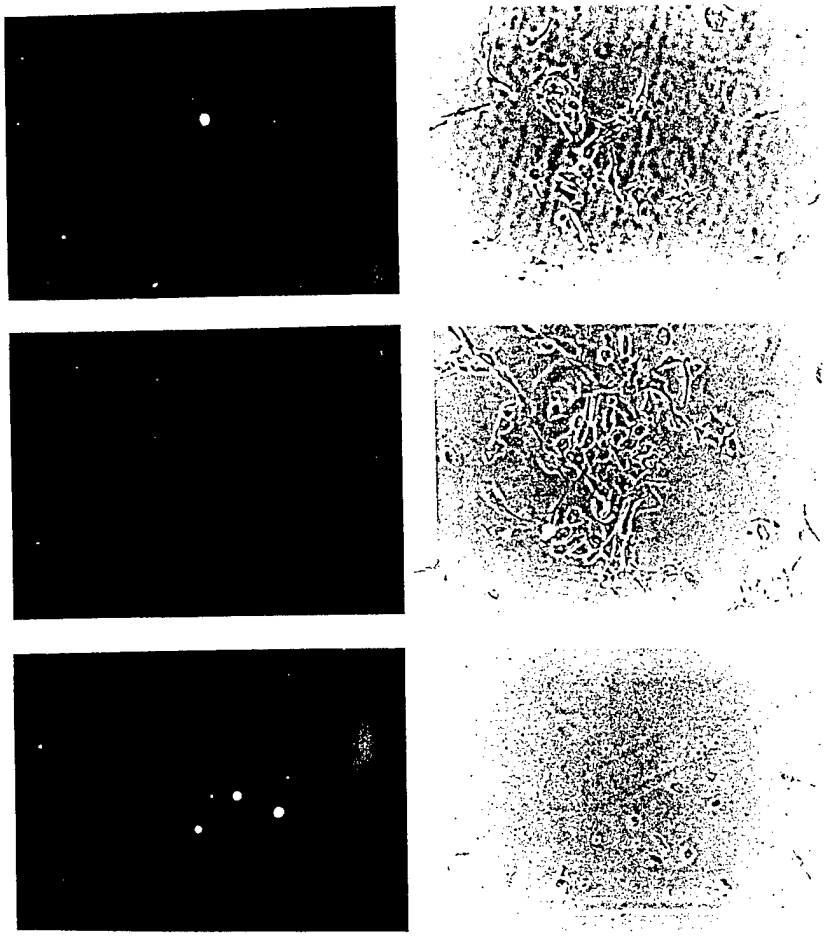


FIGURE 6A

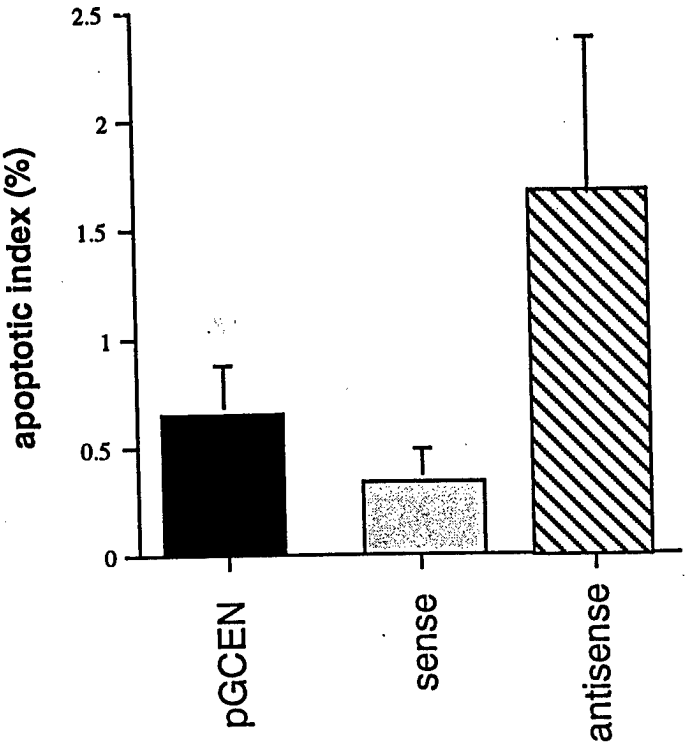
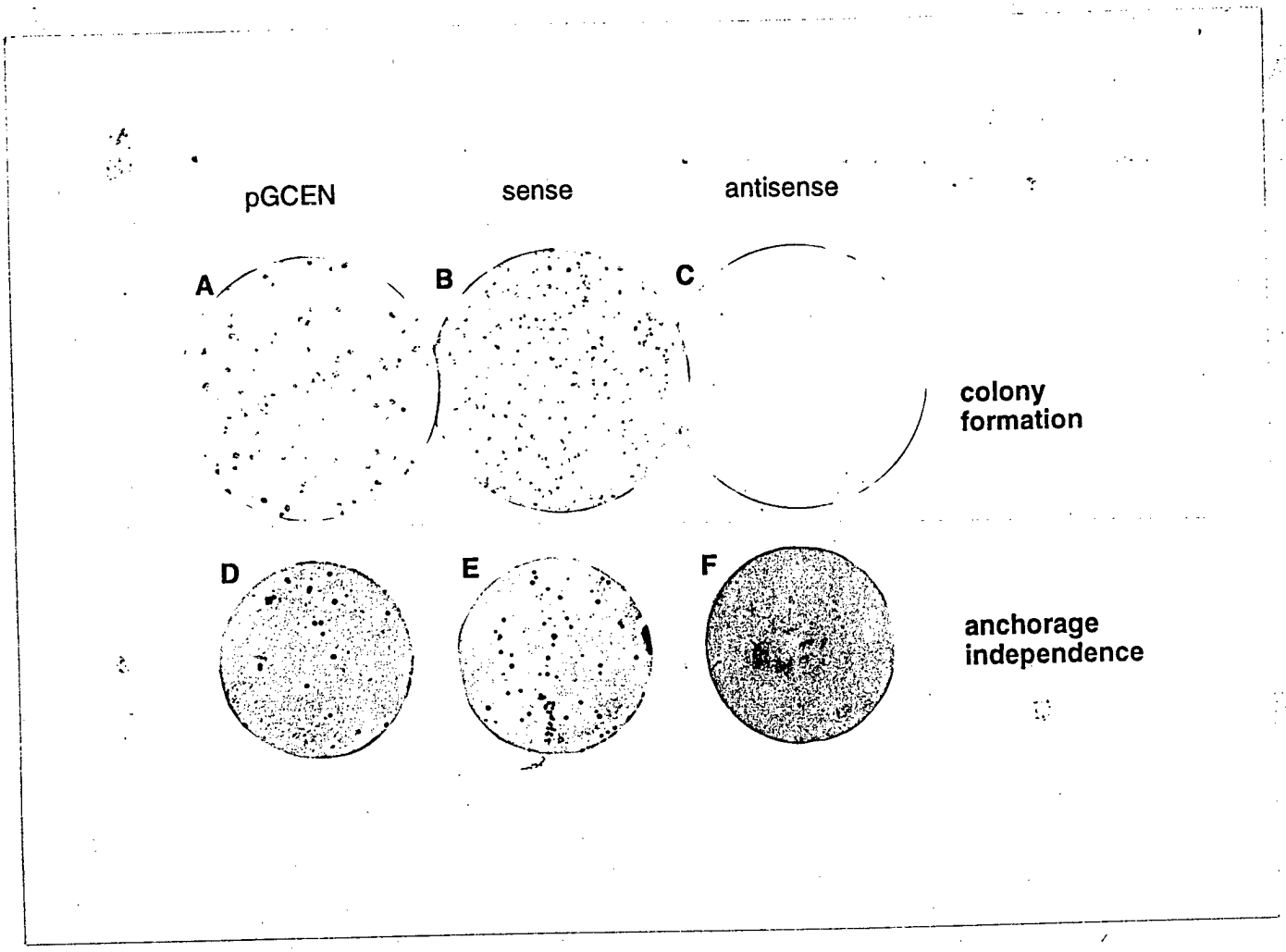


FIGURE 6B



15 79 0

0.198%

0.0375 %

>5-fold

FIGURE 7

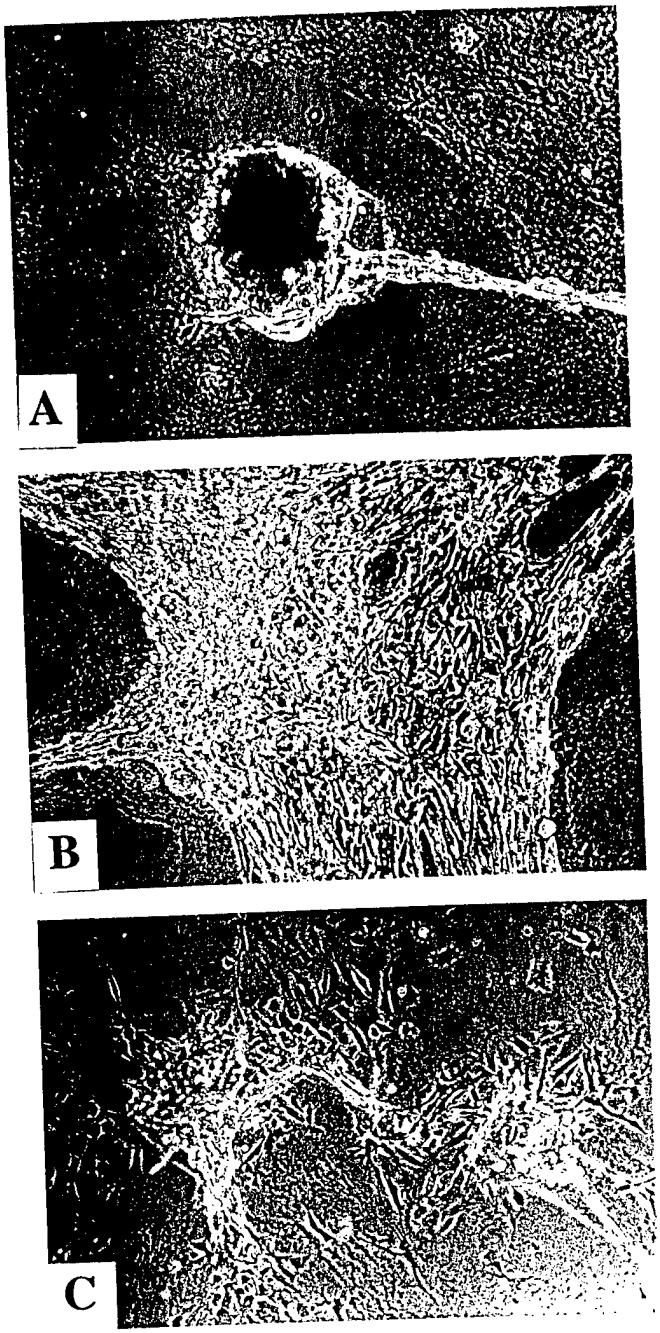


FIGURE 8

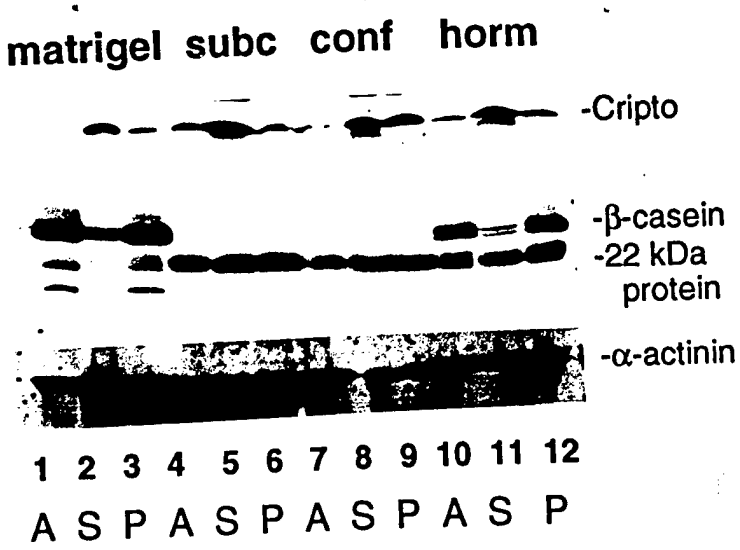

matrigel subc conf horm
-Cripto
-β-casein
-22 kDa protein
-α-actinin
1 2 3 4 5 6 7 8 9 10 11 12
A S P A S P A S P A S P

FIGURE 9

Developmental Biol, Aug 14, 1997

Specific Arrest of Cardiogenesis in Cultured Embryonic Stem cells lacking Cripto-1

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Running title: No cardiogenesis in Cr-1 KO ES cells .

Key words: homologous recombination, gene targeting, markers for ectoderm, mesoderm and endoderm, teratocarcinomas, mutant phenotype, rescue.

The molecular events of cardiac lineage specification and differentiation are largely unknown. Here we describe the involvement of a growth factor with an EGF-like domain, Cripto-1 (Cr-1), in cardiac differentiation. During embryonic development, Cr-1 is expressed in the mouse blastocyst, primitive streak, and later is restricted to the developing heart. To investigate the role of Cr-1, we have generated Cr-1-negative embryonic stem (ES) cell lines by homologous recombination. The resulting double "knockout" ES cells have selectively lost the ability to form cardiac myocytes, a process that can be rescued by reintroducing Cr-1 gene back into the Cr(-/-) cells. Furthermore, the lack of functional Cr-1 is correlated with absence of expression of cardiac-specific myosin light and heavy chain genes during differentiation. Differentiation into other cell types including skeletal muscle is not disrupted. These results suggest that Cr-1 is essential for cardiomyocyte formation *in vitro* .

Abbreviations: ES cells, embryonic stem cells; EB, embryoid body; KO, knockout; PGK, phosphoglycerokinase; tk, thymidine kinase;

INTRODUÇTION

The development of the heart is a complex process composed of two major stages: an early specification and differentiation of cardiomyocytes and later morphogenesis (Olson and Srivastava, 1996). Murine cardiac progenitor cells derive from the anterior lateral plate mesoderm that arises from the primitive streak on the 6th day of gestation (E6.5) (Olson and Srivastava, 1996). These cells differentiate, express cardiac muscle structural genes such as myosin heavy chains and atrial and ventricular myosin light chains (Kubalak *et al.*, 1994; O'Brien *et al.*, 1993), and form the primitive cardiac tube at E7.5 -8.0 (Lyons *et al.*, 1990; Morkin, 1993; Sassoon *et al.*, 1988). The newly formed cardiac tube undergoes extensive morphological changes before forming the mature heart. Through gene targeting techniques, many genes have been identified as being involved in the later processes of heart development. However, relatively little is known about the early stage of vertebrate heart development. In this report, we describe the involvement of Cripto-1 (see below) in the early stages of differentiation of cardiomyocytes.

Cripto-1 (Cr-1), encoded by the *teratocarcinoma-derived growth factor-1 (tdgf-*1) gene (Liguori *et al.*, 1996), is a growth factor with an epidermal growth factor-like motif (Ciccodicola *et al.*, 1989). Although Cr-1 folds in the characteristic EGF-like manner using its 6 cysteine residues, the resulting polypeptide is unable to bind to members of the EGF receptor family due to truncation of some sequences (Brandt *et al.*, 1994). Cr-1 is a growth factor for mammary cells by interaction with an unidentified receptor to activate intracellular components through ras/raf/MAPK pathway (Kannan *et al.*, 1997). Cr-1 and the related Cryptic gene have been assigned to a distinct family, with the proposed name, CFC (Shen *et al.*, 1997)

Both human and mouse *Cr-1* are encoded by 6 exons (exon 4 contains the EGF-like domain) producing (in mouse) a secreted protein of 171 amino acid residues (Liguori *et al.*, 1996). *Cr-1* is expressed at three main stages in development. The earliest expression of Cr-1 occurs in 4-day mouse blastocysts in both trophoblast and inner cell mass cells (Johnson *et al.*, 1994). At day 6.5, Cr-1 is present in epiblast cells and is strongly expressed at the primitive streak stage in the forming mesoderm. Then, a pattern restricted to developing heart structures occurs

(Dono *et al.*, 1993; Johnson *et al.*, 1994) with Cr-1 specifically expressed in the myocardium of the developing heart tubes in 8.5-day-old embryos and in the outflow region, conotruncus, of the heart at 9.5 -10 days gestation, when the heart develops into a functional chambered organ. After 10.5 days of development, no *in situ* hybridization signals have been detected in the embryo (Dono *et al.*, 1993). This pattern of Cr-1 expression suggests a role in the process of gastrulation, wherein the ectoderm germ layer gives rise to the mesoderm tissues including those producing the heart and somites. In addition, the highly restricted expression patterns of Cr-1 raise the possibility that Cr-1 may play a role in regulating cardiac gene expression. We used the ES model system to test this possibility.

MATERIALS AND METHODS

Construction of targeting vectors. The targeting vectors were constructed with the pPNT plasmid (Tybulewicz *et al.*, 1991). In the vector pCR-KO1, a 5-kb 5' homologous sequence containing exon 1 and 2 was inserted in front of a gene cassette encoding neomycin phosphotransferase (*neo*) gene under the control of the phosphoglycerokinase (PGK) promoter. A 3.8-kb 3' homologous fragment containing exon 6 was cloned into the *Bam*HI site downstream to the *neo*. The targeting vector pCR-KO2 was prepared by inserting the PGK-hygromycin (PGK-*hyg*) downstream of the PGK-*neo* in pCR-KO1. Negative selection to avoid random insertion was provided by the herpes simplex virus thymidine kinase (hsv-tk) gene.

Embryonic stem cell culture and selection. The ES cells were maintained in the undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblasts (MEF) feeder layers according the standard protocols. The medium used was high glucose Dulbecco's

modified Eagle's medium (DMEM) containing 15% fetal calf serum, 0.1 mM β-mercaptoethanol, 1mM sodium pyruvate, 1 x non-essential amino acids, 2 mM glutamine, 100 Units of penicillin per ml, 0.1 mg of streptomycin and 2% leukemia inhibitory factor (LIF) (conditioned media collected from confluent cultures of Chinese hamster ovary cells transfected with the LIF expression plasmid provided by permission of the Genetics Institute, Andover, MA). For the generation of Cr-1 (+/-) cells, 1x 10^7 R1 ES cells were electroporated with 30 μg linearized pCR-KO1 plasmid DNA at 300 V, 250 μF. Recombination events were selected with G418 (380 μg/ml) and gancyclovir (0.2 μM). To disrupt the second allele, Cr-1 (+/-) E31 and E40 were electroporated with linealized vector pCR-KO2 and selected in medium containing hygromycin B (400 μg/ml) and gancyclovir (0.2 μM).

Screening for recombinant clones. Genomic DNA samples were extracted from individual clones and examined for the presence of a targeted allele by PCR analysis using Taq Extender™ PCR additive system (Stratagene, San Diego, CA) or Expand™ PCR System (Boehringer Mannheim, Indianapolis, IN) with following primer pairs. The Cr-1-specific primers, 5'-ACCTGCTCTGTGTCTCCTGATTTCC-3' (p-1), which is outside (3') of the targeting vector, and 5'- CACTAGGTGACCAATCTGGTCCAAC-3' (p-2), a *neo* gene-specific primer, 5'-GCAGCCTCTGTTCCACATACACTTC-3' (p-3), and a hyg gene-specific primer, 5'-TCACGTTGCAAGACCTGCCTGAAAC-3' (p-4). The PCR conditions were as follows: 94^{0}C for 4 min; 10 cycles of 94^{0}C for 10 sec, 65^{0}C for 30 sec and 68^{0}C for 4 min, and followed by 20 cycles of 94^{0}C for 10 sec, 65^{0}C for 30 sec and 68^{0}C for 4 min with 20 sec increment for each cycle and a final extension step at 72^{0}C for 10 min. Additional PCR reaction using primers within intron 3: 5'-GTCCCTGATAGTCTCTGATATTC-3' (p-5) and 5'-GAAATGTAAGAAAAGTCATGGGG-3' (p-6) or a set of primer in *neo* gene: 5'-GTCAAGAAGGCGATAGAAGGCGATGCG-3' (p-7) and 5'-GGTGGAGAGGCTATTCGGCTATGACTG-3' (p-8) was also performed. The PCR reaction for primer p-4/p-5 and p-6/p-7 was performed by denaturing the DNA at 94^{0}C for 2 min, followed by

35 cycles of amplification: 94^{0}C for 30 sec, 55^{0}C for 30 sec, 72^{0}C for 1.5 min and a final extension step at 72^{0}C for 6 min.

RT-PCR Analysis. Total RNA (3 μg) was isolated from ES cells and EBs, treated with RNase-free DNaseI (Stratagene, San Diego, CA) and converted into cDNA using oligo(dT) as a primer. The cDNA was amplified by PCR using primers within the Cr-1 deleted region, 5'-ATCGGTCTTTCCAGTTCGTGCCTTC-3' in exon 3 and 5'-ATCTGCACAGGGAACACTTCTTGGG-3' in exon 5. A set of primers for GAPDH (Hummler *et al.*, 1996), 5'-CGTCTTCACCACCATGGAGA-3' and 5'-CGGCCATCAC GCCACAGTTT-3, were also added to the same reaction as controls. The cDNA from Day-10 EBs was amplified by PCR using specific primers for MLC2v (Miller-Hance *et al.*, 1993) and GAPDH (Hummler *et al.*, 1996). The cDNA from Day-6 EBs was amplified by PCR using brachyury -specific primers, 5'-ATCAAGGAAGGCTTTAGCAAATGGG-3' and 5'-GAACCTCGGATTCACATCGTGAGA-3', which should give a 159-bp product; GATA4 specific primers, 5'-CACTATGGGCACAGCAGCTCC-3' and 5'-TTGGAGCTGGCCTGCGATGTC-3', which should give a 143-bp product; Mef 2C specific primers (Martin *et al.*, 1993) Nkx2.5 specific primer (Biben and Harvey, 1997), 5'-TGCAGAAGGCAGTGGAGCTGGACAAGCC-3' and 5'TTGCACTTGTAGCGACGGTTCTGGAACCAG-3', which should give a 220-bp product; Egr-1 specific primers, 5'-CCGAGCTCTTCACACAACAACTTTTGTC-3' and 5'-CCGAGATCTCCCAGCTCATCATCAAAC-3', which should give a 355-bp product and GAPDH primers. The PCR reactions for Cr-1 , GAPDH, brachyury, GATA4, MEF2C and Egr-1 were performed by denaturing the DNA at 94^{0}C for 2 min, followed by 35 cycles of amplification: 94^{0}C for 30 sec, 60-65^{0}C for 30 sec, 72^{0}C for 1.5 min and a final extension step at 72^{0}C for 6 min. The PCR for MLC2V were performed by denaturing the DNA at 95^{0}C for 5 min, followed by 35 cycles of amplification: 94^{0}C for 30 sec, 70^{0}C for 30 sec, 72^{0}C for 30 sec and a final extension step at 72^{0}C for 6 min. The PCR for Nkx2.5 were amplified from cDNA converted by the Nkx2.5 specific 3'primer and performed by denaturing the DNA at 94^{0}C for 4 min, followed

by 35 cycles of amplification: 94^{0}C for 30 sec, 59^{0}C for 1 min, 72^{0}C for 1min and a final extension step at 72^{0}C for 10 min.

Differentiation of ES cells. Formation of embryoid bodies (EBs) was done by ES culture in hanging drops (Wobus *et al.*, 1991). The differentiation medium was ES culture medium containing 20% fetal calf serum but without LIF. The beating EBs were monitored by daily inspection of the culture using light microscopy. Induction of differentiation into skeletal muscle was performed by the method similar to that described by Weitzer (1995). Induction of differentiation of ES cells into neurons was performed by a method similar to that described by Bain (1995).

Stable transfection for rescue. Cells were cotransfected by lipofectamine (Gibco-BRL, Gaithersburg, MD) with a plasmid DNA containing Cr-1 sense or antisense cDNA driven by the SV40 promoter and a plasmid DNA containing puromycin resistant gene driven by the PGK promoter according to the manufacturer's recommendation. One week after the selection with 1.6 μg/ml puromycin, resistant clones were pooled, expanded and subjected to the differentiation assay.

Teratocarcinoma. 6 x 10^6 cells were injected into two subcutaneous sites of 6-week-old 129SvJ mice. Five mice were used for each cell line. The tumors were processed for histological analysis 3 weeks after the injection.

RESULTS

Targeting the Cripto-1 gene in ES cells by homologous recombination

To precisely define the function(s) of Cr-1, we have disrupted both copies of the wild-type Cr-1 alleles sequentially by homologous recombination in R1 (Nagy *et al.*, 1993) ES cells. The two targeting vectors, pCR-KO1 and pCR-KO2, caused the replacement of Cr-1 exons 3, 4 and 5

by PGK-*neo* and by PGK-*neo* -*hyg*, respectively, as determined by PCR analysis of genomic DNA (Fig. 1a-d) and Southern blotting analysis (data not shown). To further confirm the Cr-1 (-/-) mutation, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to examine whether Cr-1 mRNA was present in the clones isolated. No Cr-1 cDNA could be amplified from homozygous mutant cells while a 216-bp product was obtained from wild-type and Cr-1 (+/-) clones (Fig. 1e). In the same reaction, a 300-bp fragment of GAPDH was produced from all the samples (Fig. 1e). These tests confirmed that the Cr-1 gene was completely inactivated in the R1-derived clones selected.

Fig 1

ES cell differentiation as aggregates in hanging drops

Mouse ES cells differentiate *in vitro* into a variety of cell types including spontaneously contracting cardiac myocytes (Keller, 1995). Since Cr-1 is expressed in the developing heart during embryogenesis, we examined whether Cr-1 knockout had any effect on cardiomyocyte formation by *in vitro* differentiation analysis. Comparisons were made among several clones, including wild-type R1, Cr-1 (+/-) E31 and E40, two independent Cr-1 (-/-) ES cell lines, DE7 and DE39, obtained from transfection of Cr-1 (+/-) E31 cells, and two other independent Cr-1 (-/-) lines, DE14 and DE48, derived from Cr-1 (+/-) E40 cells. Differentiating cultures were initiated by removing ES cells from the feeder layer and forming size-controlled aggregates in hanging drop cultures (Wobus *et al.*, 1991). All cell lines tested gave rise to embryoid bodies (EBs) of similar sizes and gross morphology (data not shown). After 7 days in culture, spontaneously contracting embryoid bodies (EBs) were observed in cells derived from wild-type R1 cells. The beating activity was also observed in EBs derived from Cr-1 (+/-) E31 and E40 after 9 days in culture. The duration of continuous spontaneous contractile activity in cardiac myocytes from R1, E31 or E40 was one to two weeks. In contrast, no beating cardiac cells were observed in EBs derived from any of the four independent Cr-1 (-/-) ES clones tested (Table 1), even during culture for an additional 10 days (data not shown). Furthermore, in addition to the difference in the time for the first appearance of beating, the wild-type R1 cells gave rise to a higher percentage of beating EBs than Cr-1 (+/-) cell lines, indicating this effect was dose-dependent (Table 1). These results

Table

indicate that the formation of cardiac myocytes was completely blocked by Cr-1 mutations in ES cells. Inactivation of Cr-1 did not appear to influence cell growth of undifferentiated mouse ES cells as measured by proliferation assays (data not shown) although growth inhibition was reported for a human teratocarcinoma cell line treated with an antisense CR-1 vector (Baldassarre *et al.*, 1996)

The observed phenotype was not due to clonal variation since four independent Cr-1 (-/-) clones derived from transfection of two Cr-1 (+/-) cell lines displayed the same phenotype while none of the Cr-1 (+/+) or (+/-) cell lines was defective in cardiac myocyte formation. Nor was the phenotype a result of the drug selection, since two clones, DE29 and DE32, that remained heterozygous after the hygromycin selection formed beating EBs with similar percentages to Cr-1 (+/-) cell lines (Table 1).

Rescue of the mutant phenotype

To definitively confirm that the block to cardiac differentiation was due to the inactivation of Cr-1, we cotransfected one Cr-1 (-/-) cell line DE7 with a full-length Cr-1 sense or antisense expression plasmid together with a plasmid for puromycin resistance. Stable clones were selected for resistance to puromycin, pooled as a population of cells and examined for cardiac differentiation. Cells transfected with sense Cr-1, DE7S, were found to have regained the ability to differentiate into beating cardiomyocytes while a population of cells selected after transfection with antisense Cr-1, DE7A, were still unable to differentiate (Table 1). The expression of Cr-1 mRNA in cultures that were "rescued" for the beating phenotype was confirmed by the detection of Cr-1 mRNA only in rescued, in wt and in Cripto-1 (+/-) ES cultures (data not shown). Therefore, differentiation to cardiomyocytes is specifically blocked by the inactivation of the Cr-1 gene.

Assays for markers of cardiac differentiation

To determine where Cr-1 might be acting during the process of cardiac differentiation, we examined the expression of the myosin heavy chain (MHC) and the cardiac specific myosin light

chain 2A (MLC2A), two major contractile proteins of cardiomyocytes, in 10-day old EBs from each cell type by Western blotting. As shown in Fig. 2a, the strongest signal for MHC was detected in EBs derived from wild-type R1 cells. Cr-1 (+/-) derived EBs gave weaker signals compared to that of wild-type EBs. In contrast, expression of MHC in Cr-1 (-/-) EBs was undetectable. Similarly, expression of MLC2A was observed only in the wild-type and Cr-1 (+/-) derived EBs but not in Cr-1(-/-) EBs. Transcripts of cardiac specific myosin light chain 2V (MLC2V) were undetectable in Cr-1 (-/-) EBs as examined by RT-PCR (Fig. 2b). The expression of myosin polypeptides precedes contractile activity and so components upstream of the myosin induction pathway must be affected.

Fig. 2

As detected by RT-PCR, transcripts of precardiac mesoderm markers, transcriptional factors GATA4 (Edmondson *et al.*, 1994; Heikinheimo *et al.*, 1994), were present in Cr-1 (-/-) cell culture (Fig. 2b). The homeobox gene, Nkx2.5, is related to *tinman* in *Drosophila* where it is known to be essential for heart formation and has been implicated in the early stages of cardiac myogenesis and morphogenesis (Lyons *et al.*, 1995) mice using knockout technology. To determine if Cr-1 acted upstream of the expression of this gene, we analyzed differentiating EBs for Nkx2.5 mRNA by RT-PCR. As shown in Fig. 2b, transcripts of Nkx2.5 were detectable in Cr-1 (-/-) EBs. Egr-1, another transcriptional factor, which has been shown to be involved in stimulation of cardiac MHC expression (Gupta *et al.*, 1991), is expressed and is not affected by inactivation of Cr-1 (Fig. 2b). Expression of ErbB2 and ErbB3 in Cr-1 mutant EBs was not significantly different from wild-type EBs (Fig. 2a). This family of genes are involved in later stages of cardiac development because inactivation of ErbB2, ErbB4 and heregulin in mice leads to inhibition of cardiac muscle trabeculae formation and causes mid-gestational death (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995).

Assays for other markers of differentiation

To test the possibility that Cr-1 disrupts early stages of mesoderm differentiation, we examined the expression of an early mesoderm marker, brachyury (T), since cardiomyocytes are

mesoderm-derived. As shown in Figure 2b, brachyury transcripts were present in 6-day EBs derived from all cell lines tested, including wild-type R1, two Cr-1 (+/-) cell lines and four Cr-1 (-/-) cell lines as detected by RT-PCR. Therefore, inactivation of Cr-1 did not affect expression of this early mesoderm marker. We then evaluated the role of Cr-1 on generation of other mesoderm-derived cell types such as skeletal muscle by culturing EBs on gelatin-coated dishes. Both wild-type and mutant EBs attached to the dishes within one day and differentiating cells grew outwards. Cardiac contraction started strongly in the dense clusters of attached wild-type R1 aggregates but not in the Cr-1 (-/-) as described above, in agreement with the absence of MLC2A in outgrowths of Cr(-/-) EBs (data not shown). Small myotubes started to form in both wild-type and mutant cell cultures after two weeks in culture and myotubes continued to grow into large skeletal myotubes (Fig. 3a) that contracted frequently on Day 20-25. The formation of skeletal muscle was further confirmed by expression of a skeletal specific gene, myogenin, as detected by RT-PCR (data not shown).

Differentiation into other mesoderm and other cell types was demonstrated by the generation of teratocarcinomas, which form a wide range of recognizable differentiated cell types. To examine differentiation *in vivo*, wild-type R1 cells and Cr-1(-/-) DE39 cells were injected (s.c.) into 129 SvJ mice. After 3 weeks, tumors formed from both the mutant cells and wild type cells and histological examination indicated that the teratocarcinomas contained many cell types. Dermis and squamous epidermis (Fig. 3c), cartilage (Fig. 3d) and epithelioid organs (Fig. 3e) were observed in teratocarcinomas derived from both cell lines. Therefore, inactivation of Cr-1 did not affect differentiation of other mesoderm cell types in spite of the inability to differentiate into cardiomyocytes. Fig. 3

To test if the mutant cells can differentiate into neurons, an ectoderm-derived cell type, the EBs were treated with trans-retinoic acid which enhanced neural differentiation and then plated on gelatin-coated dishes. One week after plating, cells with neuron-like morphology were found in the culture. Fig. 3b shows a neurite outgrowth in the Cr-1 (-/-) cell culture two weeks after plating.

To determine if inactivation of Cr-1 affects the differentiation of endoderm, the expression of alpha-fetoprotein (AFP), a marker for visceral endoderm cells, and laminin, a marker for parietal endoderm cells among other tissues, were not significantly different in mutant EBs at day 8 from those in wild-type cells as determined by Western blotting analysis (Fig. 2a). Thus, Cr-1 is not required for differentiation of endoderm.

DISCUSSION

Functional and biochemical differentiation is readily studied using ES cells because of their ability to differentiate into a range of cell types in culture and into teratocarcinomas in adult hosts. The *in vitro* model of cardiogenesis using ES cells has been well characterized and the temporal stages in heart differentiation and function have been mapped, although spatial signals and morphogenesis are lacking in this model (Fassler *et al.*, 1996; Maltsev *et al.*, 1993; Metzger *et al.*, 1996; Miller-Hance *et al.*, 1993; Wobus *et al.*, 1995).

Based on our results of *in vitro* and *in vivo* differentiation of ES cells, we concluded that disruption of Cr-1 results in a specific defect in cardiac differentiation but there is no detectable defect in differentiation of other mesodermal, endodermal and ectodermal cell types. These results emphasize a potentially important function for Cr-1 in cardiomyocyte differentiation at an early stage of cardiac development, which is consistent with predictions based on the expression pattern of Cr-1 during mouse embryogenesis.

Several genes expressed in precardiac mesoderm, such as Nkx2.5 (Lyons *et al.*, 1995), Mef2C (Edmondson *et al.*, 1994) GATA4 (Heikinheimo *et al.*, 1994) eHAND and dHAND were predicted to be responsible for the commitment of mesodermal cells to the cardiac lineage. However, inactivation of these genes in mice did not affect differentiation of cardiomyocytes (Lyons, 1996; Olson and Srivastava, 1996). All of the transcription factors that we assayed that were shown to be involved in cardiac development were transcribed in Cripto null ES cell cultures. Therefore Cripto acts independently (or downstream) of these genes. Other than bone morphogenetic protein (BMP4) in chicks (Schultheiss *et al.*, 1997) and hepatocyte growth factor

(HGF) in mouse (Rappolee *et al.*, 1996) very little is known about how growth factors affect early cardiogenesis. We describe here for the first time that inactivation of the growth factor gene, Cr-1, results specifically in defective early differentiation of cardiomyocytes. This phenotype was specific to cardiogenesis because no other major tissue type was absent. The abrogation of the Cripto-1 gene was the cause of the phenotype because the re-expression of exogenous Cripto-1 in the "knockout" cells reversed the mutant phenotype. It is possible that Cripto-1 may be a master switch gene that activates the myosin and actin genes in the cardiomyocyte, such as by activation of the Serum Response Factor (SRF) gene. This factor is one of several factors known to upregulate the activity of the myosin, actin and other muscle specific genes (Donoviel *et al.*, 1996). The unique specificity of Cripto-1 for cardiac cell differentiation is unusual, because most factors activate both cardiac and skeletal muscle lineages.

To devise strategies for cardiac muscle cell transplantation in heart disease treatments (Field, 1993), it will be helpful to define how specific growth factors such as Cr-1 promote cardiac lineages. Thus, these Cr-1 (-/-) and other targeted ES cell lines will provide a good system for studying specification and differentiation of the cardiac lineage and may provide information for therapy programs aimed towards regeneration of myocardium.

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FIGURE LEGENDS

Figure 1 Generation of Cr-1 (-/-) cells. (a) and (b) Strategies for disrupting Cr-1 alleles. Exons are depicted by the open boxes and numbered. B represents *Bam*HI. (c) and (d) Genotyping of cell lines. The primer p-3 and p-1 amplified a 4.2 kb product in Cr-1 (+/-) clones and 4.2-kb and 6.8-kb products in Cr-1 (-/-) cells (under neo). The p-1 and p-2 amplified a 4.8-kb product from Cr-1 (+/+) and (+/-) cells (under wt). The p-4 and p-1 gave a 5.2-kb product only in the Cr-1 (-/-) cells (under hyg). The p-5 and p-6 amplified a 242-bp product from Cr-1 (+/+) or (+/-) cells while the p-7 and p-8 amplified a 739-bp product from Cr-1 (+/-) and (-/-) cells. (*e*) RT-PCR verification of Cr-1 mutations.

Figure 2 (a) Western blot analysis of EBs. EBs derived from indicated cell lines at the specific day were detected for the MHC by a monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, NICHD, NIH), for MLC2A by a rabbit antiserum (a kind gift from Dr. K. Chien, Dept. of Medicine, University of California at San Diego), for ErbB2 and ErbB3 by rabbit antibodies (Santa Cruz Biotechnology Inc. CA), and for AFP and laminin by rabbit antisera. For a control, the blot was reprobed with antibody against β-actin or α-actinin. Signals for binding of the antibodies were detected by the enhanced chemiluminescence system (ECL, Amersham Corp. Aylesbury, U.K.). (b) RT-PCR detection of MLC 2V and GAPDH in Day-10 EBs; brachyury, GATA4, Mef2C, Nkx2.5, Egr-1 and GAPDH in Day-6 EBs.

Figure 3. *In vitro* and *in vivo* differentiation of Cr-1 (-/-) cells. (a) Differentiation of Cr-1 (-/-) cells into skeletal muscle. EBs derived from the Cr-1 (-/-) cell line DE39 were formed by hanging drops and transferred to gelatin-coated dishes on day 5. Strongly contracting myotubes around the attached EBs were observed after culture for an additional two weeks. (b) Differentiation of Cr-1 (-/-) cells into neurons. The EBs were also treated with 5 x 10^{-7} M retinoic acid on days 5-8 and transferred to gelatin-coated dishes after partial dissociation with trypsin-EDTA. Neurons were

formed after plating. (c, d, e), Stained sections (hematoxylin and eosin) of teratocarcinomas derived from Cr-1 (-/-) ES cells. The tumors derived from Cr-1 (-/-) DE39 cells were placed in Bouin's fixative, embedded in paraffin and sectioned at 5 μm. Sections with dermis and squamous epidermis (c), cartilage (d) and epithelioid organs (e). Bar = 100μm.

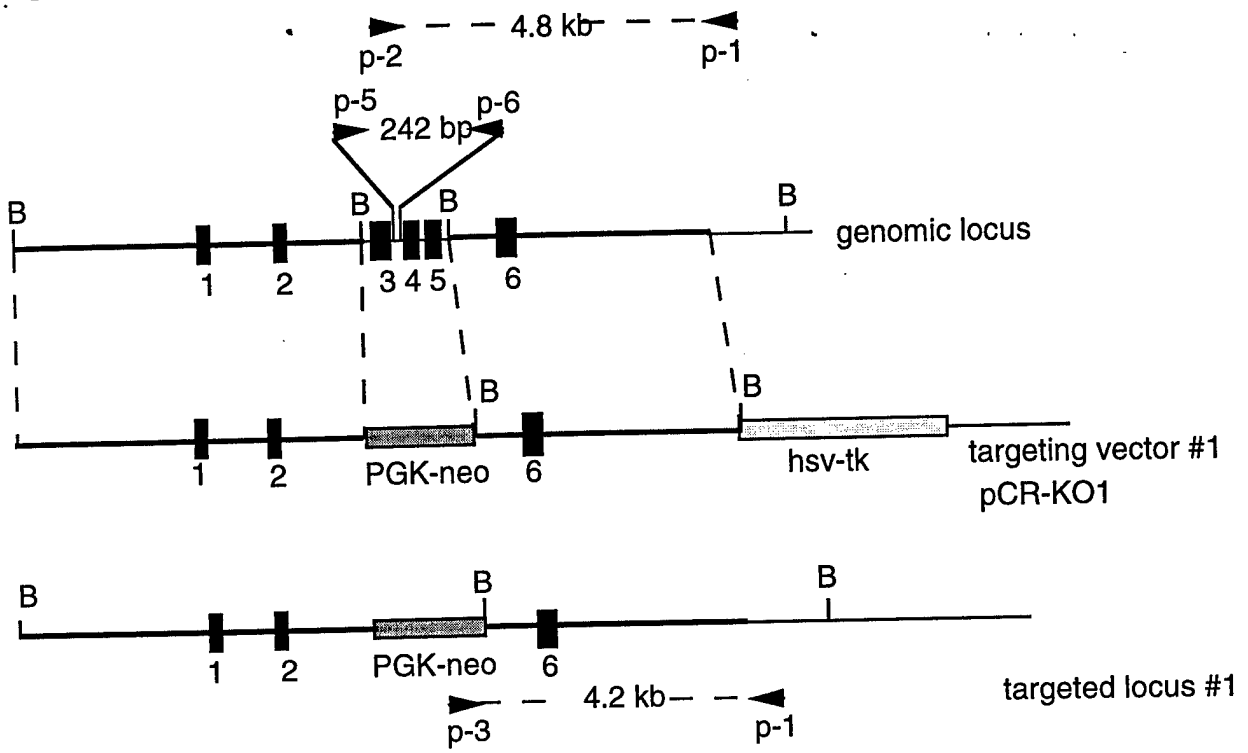
Table 1 Inhibition of cardiomyocyte differentiation in Cr-1 (-/-) cells

Cells	No. of EBs	Percentage of EBs with beating cardiomyocyte								
		D 6	D 7	D 8	D 9	D 10	D 11	D 12	D 13	D 14
R1 (+/+)	55	0	32.7	52.7	58.2	38.2	32.7	14.5	9.0	7.2
E31 (+/-)	46	0	0	0	4.3	10.9	8.7	2.2	2.2	2.2
E40 (+/-)	49	0	0	0	18.4	30.6	34.7	24.5	24.5	16.3
DE29 (+/-)	37	0	0	0	5.4	13.5	10.8	10.8	8.1	5.4
DE32 (+/-)	48	0	0	14.5	20.8	29.2	14.6	12.5	12.5	10.4
DE7 (-/-)	52	0	0	0	0	0	0	0	0	0
DE14 (-/-)	43	0	0	0	0	0	0	0	0	0
DE39 (-/-)	46	0	0	0	0	0	0	0	0	0
DE48 (-/-)	34	0	0	0	0	0	0	0	0	0
DE7S	41	0	0	0	2.4	4.9	7.3	7.3	4.9	2.4
DE7A	45	0	0	0	0	0	0	0	0	19 0

EBs were cultured from wild-type ES cells, R1(+/+), from cell lines with one Cr-1 allele mutated, E31 and E40 (+/-), from cell lines with both Cr-1 alleles mutated, DE7 (-/-) and DE39 (-/-) (derived from E31), DE14 (-/-) and DE48 (-/-) (derived from E40). Cells remained heterozygous after the hygromycin selection, DE29 (+/-) and DE 32 (+/-) were also tested for the ability and timing of beating cardiomyocyte formation.

DE7(-/-) cells transfected with a plasmid containing Cr-1 sense, population DE7S, or antisense Cr-1 cDNA, population DE7A, were monitored for beating cardiomyocyte aggregates from Day 6 to 14. The rescue of beating in Cr-1(-/-) cells occurred only in the sense-expressing cultures. Similar results were found in more than three independent experiments. D, Day.

a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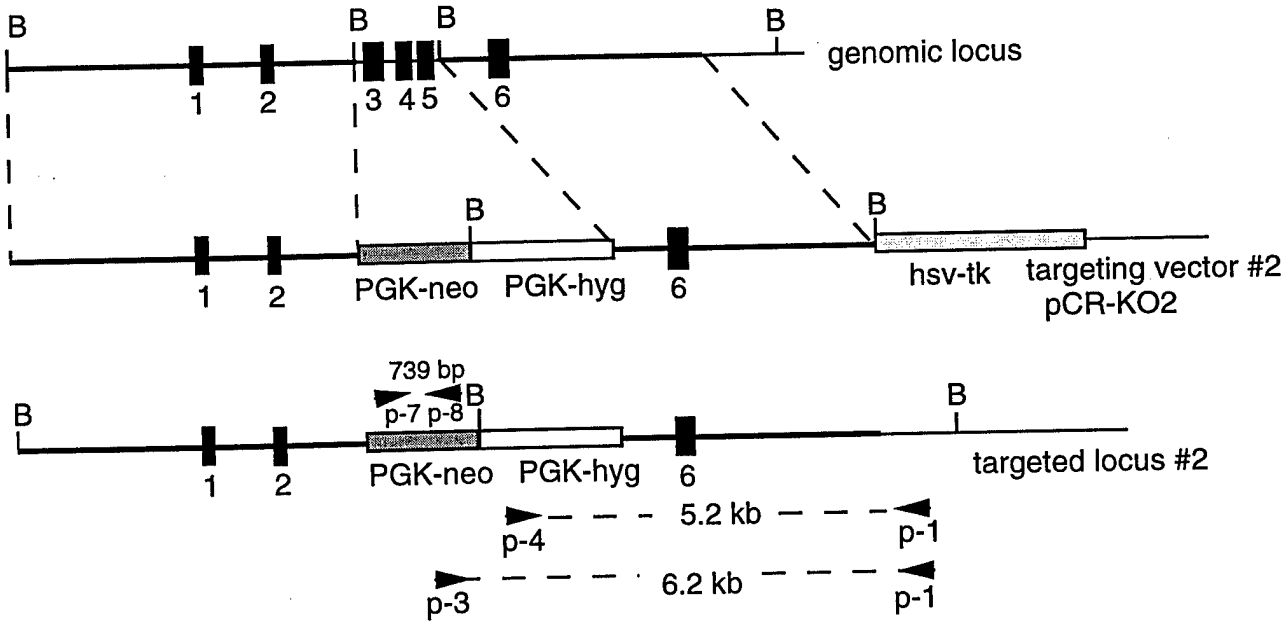
b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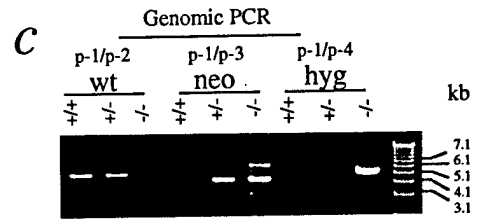
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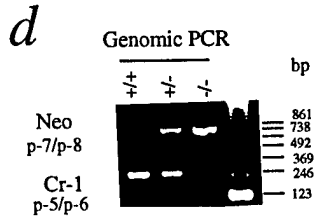
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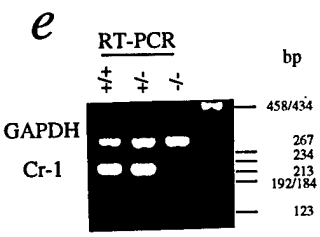
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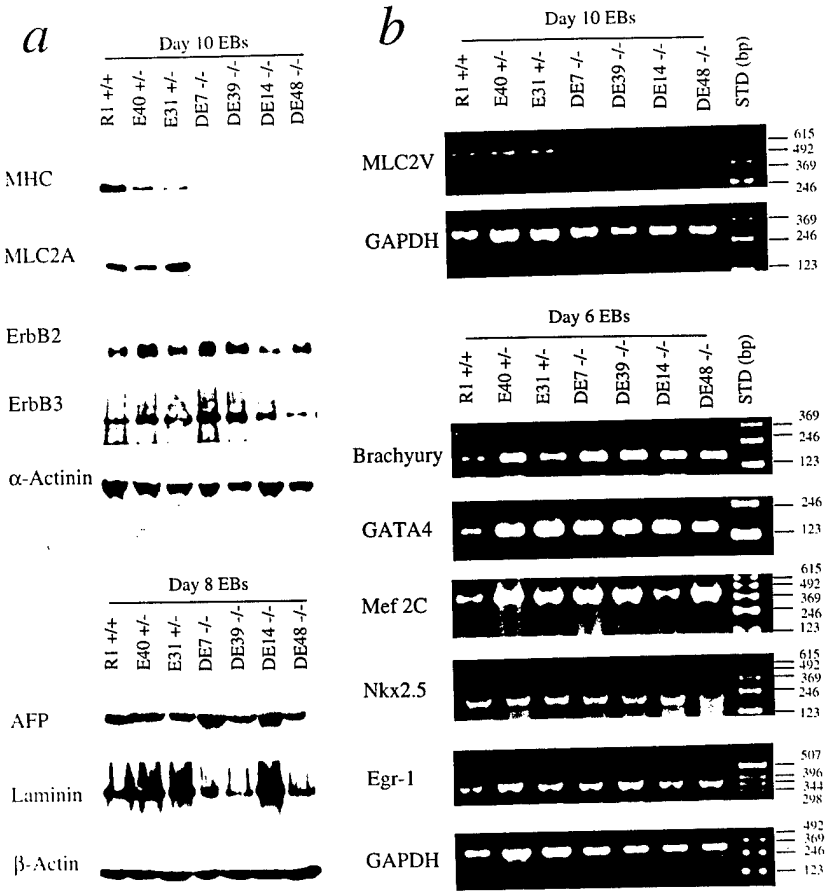
a
Day 10 EBs
R1 +/+
E40 +/-
E31 +/-
DE7 -/-
DE39 -/-
DE14 -/-
DE48 -/-
MHC
MLC2A
ErbB2
ErbB3
α-Actinin
Day 8 EBs
AFP
Laminin
β-Actin
b
Day 10 EBs
STD (bp)
MLC2V
GAPDH
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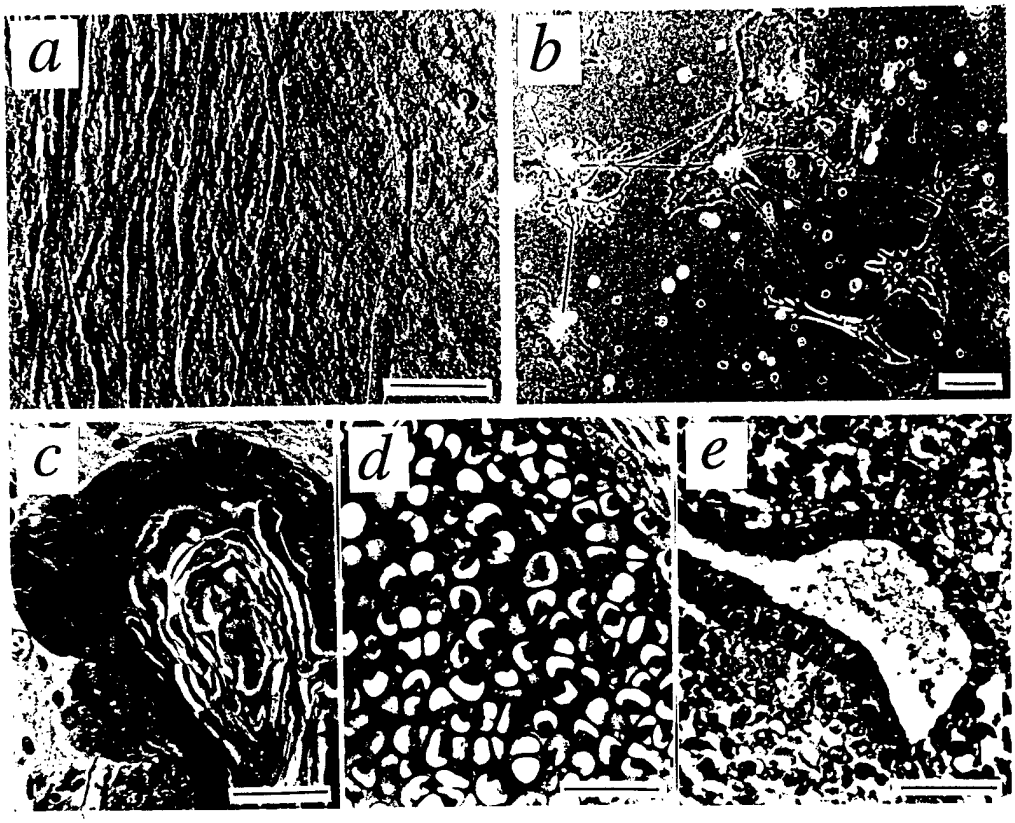
Mef 2C
Nkx2.5
Egr-1
507
396
344
298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