Stem cell defects in parthenogenetic peri-implantation embryos

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SUMMARY

Mouse embryos containing only maternal chromosomes (parthenotes) develop abnormally in vivo, usually failing at the peri-implantation stage. We have analyzed the development of parthenote embryos by using an inner cell mass (ICM) outgrowth assay that mimics peri-implantation development. ICMs from normal embryos maintained undifferentiated stem cells positive for stage-specific embryonic antigen-1 and Rex-1 while differentiating into a variety of cell types, including visceral endoderm-like cells and parietal endoderm cells. In contrast, ICMs from parthenotes failed to maintain undifferentiated stem cells and differentiated almost exclusively into parietal endoderm. This suggests that parthenote ICMs have a defect that leads to differentiation, rather than maintenance, of the stem cells, and a defect that leads to a parietal endoderm fate for the stem cells. To test the hypothesis that the ICM population is not maintained owing to a lack of proliferation of the stem cells, we investigated whether mitogenic agents were able to maintain the ICM population in parthenotes. When parthenote blastocysts were supplied with the insulin-like growth factor-1 receptor (Igf-1r) and insulin-like growth factor-2 (Igf-2), two genes not detectable in parthenote blastocysts by in situ hybridization, the ICM population was maintained. Similarly, culture of parthenote blastocysts in medium conditioned by embryonic fibroblasts and supplemented with the maternal factor leukemia inhibitory factor maintained the ICM population. However, once this growth factor-rich medium was removed, the parthenote ICM cells still differentiated predominantly into parietal endoderm. These data suggest that the parthenote ICM cells have both a proliferation defect and a cell fate defect owing to misregulation of genes critical to growth and differentiation, and that these defects are responsible, in part, for the abnormal development of peri-implantation parthenote embryos.

Key words: parthenote, gametic imprinting, peri-implantation development, insulin-like growth factor-2, insulin-like growth factor-1 receptor, stem cell, mouse

INTRODUCTION

Normal mammalian development requires genomic contributions from both the mother and the father. Mammalian parthenotes do not survive to term, because the maternal and paternal chromosomes of mammals are not equivalent (McGrath and Solter, 1984). Uniparental duplications of regions of some chromosomes are lethal or detrimental to the embryo. Eleven such regions have been found (Searle and Beechey, 1978); in a few of these regions, genes have been identified that are expressed from only one allele, either maternal or paternal. For the embryo to distinguish between the two alleles, at some point in gametogenesis the genes must be marked as maternal or paternal. This gametic imprint causes a functional difference in the gene product, such as transcription of the gene (Barlow, 1994). The genes that have been identified as imprint have a wide range of functions, from splicing factors, such as Surtpt and (potentially) Sp2, to growth factors, such as insulin (Ins1 and Ins2) and Igf-2, to genes that are functional as RNAs, such as H-19 and Xist (reviewed by Bartolomei, 1994).

Gametic imprinting, presumably, is the main cause of uniparental failure in development, although nonimprinted genes, such as insulin-like growth factor-1 receptor (Igf-1r), may be misregulated in parthenogenetic embryos (Rappolee et al., 1992). Parthenogenetic embryos fail in development in a characteristic fashion. The most advanced parthenogenetic embryos survive to the early limb bud stage. These embryos have little extraembryonic tissue and almost no trophoblast (Kaufman et al., 1977). Most parthenogenetic embryos develop into a disorganized mass of parietal endoderm (PE) cells (Sturm et al., 1994). Although supplying parthenogenetic stem cells (Allen et al., 1994) or embryos (Spindle, A., Sturm, K., Flannery, M., Meneses, J., Wu, K. and Pedersen, R., unpublished data) with trophoblast allows a higher percentage of the embryos to reach the early limb bud stage, the embryos still die, showing that both genomes are needed for normal embryonic development. Studies with chimeras show that both genomes are needed in at least some cells of the embryo. In chimeras between normal (zygotic) embryos and parthenotes, parthenogenetic cells are excluded from day 6.5 p.c. trophoblast, but not from the inner cell mass (ICM) derivatives in the embryo proper (Clark et al., 1993). At midgestation, parthenogenetic cells are excluded from parts of the embryo proper, including skeletal muscle, liver and pancreas (Fundele et al., 1990). Therefore, parthenotes fail not only because they develop a small trophoblast, but also because of some cell-autonomous defects.
In this study, we have characterized the development of early parthenogenetic embryos and investigated the causes of parthenote failure by using an assay that mimics peri-implantation development in vivo. We characterized two defects of parthenote ICM cells, a lack of maintenance of the ICM cells and a differentiation of the ICM cells into a disproportionate number of PE cells. We also investigated whether treatment with mitogenic agents present in the maternal environment or in normal embryos, but not parthenote embryos, was able to rescue these defects.

MATERIALS AND METHODS

**Materials**

(C57BL/6J x CBA/J)F1 mice (B6CBA) were obtained from Jackson Laboratories (Bar Harbor, ME). Pregnant mares' serum gonadotropin, insulin-like growth factor-2 (Igf-2), biotin-labeled sheep anti-rabbit immunoglobulins and biotin-labeled goat anti-mouse IgM were purchased from Sigma Chemical Co. (St Louis, MO). Human chorionic gonadotropin (hCG) was obtained from Serono (Randolph, MA). Dulbecco's modified Eagle's medium (DME) was prepared by the Cell Culture Facility (University of California, San Francisco). Fetal bovine serum was obtained from Hyclone (Logan, UT). Human fibronectin (FN) was purchased from Boehringer Mannheim (Indianapolis, IN). Rabbit anti-mouse laminin (LN) immunoglobulins were purchased from Collaborative Research (Lexington, MA). Fluorescein-conjugated swine anti-rabbit immunoglobulins were purchased from Dakopatts (Santa Barbara, CA). Fluorescein-labeled streptavidin was purchased from Vector Laboratories, Inc. (Burlingame, CA). Monoclonal antibody to stage-specific embryonic antigen-1 (SSEA-1) was derived from a hybridoma provided by American Type Culture Collection (MC-480) (Solter and Knowles, 1978).

**Embryo collection and parthenogenetic activation**

Mice were housed in a pathogen-free mouse room on a standard 12 hour light/dark cycle. Mice were superovulated by intraperitoneal injection of 10 units of pregnant mares' serum gonadotropin, insulin-like growth factor-2 (Igf-2), biotin-labeled sheep anti-rabbit immunoglobulins and biotin-labeled goat anti-mouse IgM were purchased from Sigma Chemical Co. (St Louis, MO). Human chorionic gonadotropin (hCG) was obtained from Serono (Randolph, MA). Dulbecco's modified Eagle's medium (DME) was prepared by the Cell Culture Facility (University of California, San Francisco). Fetal bovine serum was obtained from Hyclone (Logan, UT). Human fibronectin (FN) was purchased from Boehringer Mannheim (Indianapolis, IN). Rabbit anti-mouse laminin (LN) immunoglobulins were purchased from Collaborative Research (Lexington, MA). Fluorescein-conjugated swine anti-rabbit immunoglobulins were purchased from Dakopatts (Santa Barbara, CA). Fluorescein-labeled streptavidin was purchased from Vector Laboratories, Inc. (Burlingame, CA). Monoclonal antibody to stage-specific embryonic antigen-1 (SSEA-1) was derived from a hybridoma provided by American Type Culture Collection (MC-480) (Solter and Knowles, 1978).

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**Embryo collection and parthenogenetic activation**

Mice were housed in a pathogen-free mouse room on a standard 12 hour light/dark cycle. Mice were superovulated by intraperitoneal injection of 10 units of pregnant mares’ serum followed 48 hours later by intraperitoneal injection of 5 units of hCG. Fertilized embryos were obtained by mating the superovulated female mice to B6CBA males. The embryos were cultured in TE medium (Spindle, 1990) under a 1:5 dilution of rabbit anti-mouse antibody for 10 minutes at 37°C, washed, then incubated in rat serum (Harlan Bio-products, Indianapolis, IN) for 30 minutes. The lysed trophoblast cells were removed and the ICMs were cultured individually on a substrate of 50 μg/ml FN in T-extra medium overlaid with mineral oil.

**Immunocytochemical analysis**

Immunocytochemical analysis was performed essentially as described previously (Sutherland et al., 1993). The dilutions of primary antibodies used were as follows: anti-LN, 1:25; anti-SSEA-1, no dilution. IgM (Chemicon, Temecula, CA) and rabbit IgG were used as negative controls when appropriate. Cultures were incubated with the appropriate secondary antibody as follows: For SSEA-1, biotin-labeled goat anti-mouse IgM, 1:1000 dilution; for LN, fluorescein-labeled swine anti-rabbit IgG, 1:50 dilution. Streptavidin labeled with fluorescein (1:1000 dilution) was used for detection of SSEA-1.

**Apoptosis detection**

Apopototic cells in day 2 ICM cultures were labeled with digoxigenin-dUTP by using terminal deoxynucleotidyl transferase exactly as described in the ApoTag kit from Oncor (Gaithersburg, MD) and detected with fluorescein-labeled streptavidin. Apoptotic vesicles were counted under fluorescent light.

**In situ hybridization**

Digoxigenin-labeled probes were prepared with linearized sense and anti-sense DNA templates by using a kit supplied by Ambion (Austin, TX). Whole-mount in situ hybridization was performed essentially as described by Wilkinson (1992), with the following modifications. For easier processing of preimplantation embryos, they were placed in baskets made by cutting off the bottom of a 0.5 ml Eppendorf tube and melting a small piece of nylon membrane with 20 μm pores onto it (Fisher, Pittsburgh, PA). Instead of proteinase K treatment, we used three washes of 30 minutes each in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 50 mM Tris-HCl) (Bursdal et al., 1993). For detection of the alkaline phosphatase-labeled antibody, the samples were incubated with BM Purple (Boehringer-Mannheim) containing 2 mM levamisole. Normal and parthenogenetic embryos were processed simultaneously throughout the procedure, including time in the detection reagent.

**DNA injections**

DNA was injected into one of the nuclei of 2-cell embryos according to the protocol for injection into the pronucleus of 1-cell embryos as described by Hogan et al. (1994). DNA was injected at a concentration of 50 μg/ml in sterile 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA, β-galactosidase staining was done either with 5-bromo-4-chloro-3-indolyl β-D-galactoside after fixation (Vernet et al., 1993) or in live embryos with Imagen Green substrate from Molecular Probes (Eugene, OR).

ICM cell numbers were counted at the blastocyst stage by isolating ICMs by immunosurgery and staining the nuclei with 1 μg/ml Hoechst 33258 in phosphate-buffered saline, pH 7.0, for 10 minutes. The ICM nuclei were counted under ultraviolet light with epifluorescence.

**Constructs**

All expression constructs were based on pECE, which contains the simian virus (SV) 40 promoter (Ellis et al., 1986). Igf-1r and Instr cDNAs were kindly provided by William Rutter. The Snrpn probe was kindly provided by Michael Lerner. The Rex-1 cDNA was kindly provided by Lorraine Gudas (Rogers et al., 1991).
Parthenote stem cell defects

Transfection protocol
Preimplantation embryos were transfected essentially as described by Cross et al. (1995). Precompaction 4-cell embryos were treated briefly with acid Tyrode’s solution to remove their zonae pellucidae, washed with DME with 10% serum and incubated for 2 hours. DNA (2 μg of the Igf-1r expression construct and 1 μg of the RSV-βGal expression construct) was mixed with Lipofectin (Gibco/BRL, Gaithersburg, MD) as directed. After 8 hours in a 37°C incubator, the embryos were washed with TE medium and cultured in medium with or without Igf-2 at a concentration of 100 ng/ml.

RESULTS
Parthenote ICM outgrowths lack undifferentiated stem cells
We examined the development of parthenotes in vitro by using ICMs isolated at the late blastocyst stage by immunosurgery as a culture model of peri-implantation development (Behrendtson et al., 1994). After 3 days in culture, three cell types could be identified by morphological and molecular
criteria: PE cells, ICM cells and cells that did not meet the criteria of either PE or ICM cells, including visceral endoderm-like cells (Behrendtsen et al., 1994). The ICM cells expressed two molecular markers: SSEA-1, a complex cell surface carbohydrate (Solter and Knowles, 1978) and Rex-1, a transcription factor (Rogers et al., 1991). Although most of the normal ICM cultures contained cells positive for SSEA-1, as detected immunocytochemically, few of the parthenote ICM cultures did (Fig. 1A-F). Whole-mount in situ hybridization demonstrated that normal ICM cultures also contained cells that expressed Rex-1 mRNA, whereas most parthenote cultures did not (Fig. 1G-J). These results suggest that the ICM cells are not maintained in parthenotes.

**Parthenote ICMs differentiate predominantly into PE**

PE are migratory cells that have a distinct morphology and secrete LN (Timpl et al., 1979; Behrendtsen et al., 1994), as well as secreted protein acidic and rich in cysteine (SPARC) (Mason et al., 1986; Behrendtsen et al., 1994) and vimentin (Lane et al., 1983; Behrendtsen et al., 1994). These cells do not express SSEA-1, Rex-1 (Fig. 1), or a-fetoprotein (Behrendtsen et al., 1994). Fig. 2A-D shows immunocytochemical analysis with an anti-LN antibody of representative cultures 3 days after isolation of ICMs. The normal ICM cultures contained at least three cell types: small, tightly packed cells that were negative for LN, large, flat cells that expressed low amounts of LN and small, individual PE cells that expressed high amounts of LN (Fig. 2A,B). Parthenote ICM outgrowths were different from the normal ICM outgrowths: most of the cells derived from parthenote ICMs were PE cells and few of the outgrowths contained cells with no or low expression of LN (Fig. 2C,D). We counted the total number of cells as well as the number of PE cells (defined as cells expressing high levels of LN and displaying PE morphology) and found that 80% of the cells in parthenote ICM outgrowths were PE, whereas 41% of the cells in normal ICM outgrowths were PE (Fig. 2E). In fact, after 3 days, 36% of the parthenote ICM cultures contained only PE cells, whereas only 4% of the normal ICM cultures contained only PE cells.

In a separate set of experiments, we photographed ICM outgrowths on days 1, 2, and 3 and counted the total number of cells and the number of PE cells (based on morphology alone). The total number of outgrowth cells in normal ICM cultures more than doubled from day 2 to 3, whereas the total number of outgrowth cells in parthenote ICM cultures increased only slightly (Fig. 2F). At day 2 the parthenote cultures contained about 50% PE cells. However, at day 3, most of those outgrowth cells had differentiated to PE. These data show that by day 3, most normal ICMs have differentiated into a variety of cell types, whereas parthenote ICMs have differentiated predominantly into PE.

The increase in the proportion of PE cells and the loss of ICM did not appear to be due to the death of the ICM or to cells of another lineage, because no difference in apoptosis was seen in normal and parthenote ICM cultures at day 2 (apoptotic bodies in normal cultures, 16.2±2.9, n=25; in parthenote cultures, 12.0±2.0, n=20; mean ± s.e.m.).

**Mitogenic agents increase the ICM population in outgrowths**

The results of the experiments described above suggested that parthenote ICMs have two developmental defects: they do not maintain an ICM population and they differentiate predominantly into PE. We first addressed the lack of ICM maintenance.

We hypothesized that the parthenote ICM cells do not proliferate as much as the normal ICM cells, leading to fewer ICM cells at the outgrowth stage. Previous data from this laboratory have shown that a mitogenic pathway, the Igf-1r/Igf-2 pathway, is misregulated in parthenotes (Rappolee et al.,

Fig. 2. Parthenote ICMs differentiate predominantly into PE. Normal (A,B) and parthenote (C,D) ICM outgrowths were stained with anti-LN antibody. (A,C) Phase-contrast photomicrographs; (B,D) fluorescent photomicrographs. Filled arrows point to PE cells; open arrows point to non-PE cells. Bar indicates 50 μm. (E) Parthenote ICM cultures (striped bar) had a higher percentage of total cells that were PE than did normal ICM cultures (solid bar). Normal, 41.1±7.9%; parthenote, 80.3±3.3%; P<0.001. Total cell number: Normal, 60.1±8.5; parthenote, 45.0±5.5. PE cell number: Normal, 28.2±4.7, n=19; parthenote, 38.0±4.7, n=27. (F) Total and PE cell counts at days 1, 2, and 3 in normal (solid line) and parthenote (dashed line) outgrowth cultures.
1992). We used whole-mount in situ hybridization to examine the expression of Igf-1r and Igf-2, as well as that of another growth factor receptor, the insulin receptor (Insr), in normal and parthenote blastocysts. Both Igf-2 and Igf-1r were expressed in normal blastocysts, but in three separate experiments, neither was detected in parthenotes (Fig. 3). To control for sample-to-sample variability, we isolated and processed the blastocysts at the same time and developed them in substrate for the same amount of time. Insr mRNA was readily detectable in both parthenote and normal blastocysts (Fig. 3). No signal was present when Igf-2, Igf-1r, or Insr sense probes were used (data not shown).

Next, we attempted to increase the ICM population by supplying parthenotes with the Igf-1r/Igf-2 pathway. We introduced Igf-1r into the parthenotes by microinjection of an expression construct containing human Igf-1r into one nucleus of the 2-cell embryo. Injection at the 2-cell stage gives much higher expression during preimplantation development than injection at the 1-cell stage (Miranda et al., 1993). Embryos were co-injected with circular plasmid (50 ng/μl) containing the SV40 promoter with or without human Igf-1r cDNA and another plasmid containing βGal. We also injected some embryos with an expression construct for an imprinted gene, Snrpn, which is expressed only from the paternal allele (Reed and Leff, 1994), both as a control for Igf-1r specificity and to determine if it had any effect on cell proliferation in parthenotes. Of the injected embryos, approximately 50% developed to the blastocyst stage. Of these, 90% expressed the marker gene βGal in at least one cell, as detected by accumulation of fluorescent product in living blastocysts. The number of ICM cells at the blastocyst stage was determined by performing immunosurgery, after which the ICM nuclei were stained with Hoechst 33258 and counted. Parthenote embryos injected with Igf-1r and treated with Igf-2 had significantly more ICM cells at the blastocyst stage than did those injected with Igf-1r but not treated with Igf-2 (Fig. 4). Injection of the expression construct containing the promoter alone or the Snrpn expression construct, with or without treatment with Igf-2, did not significantly affect the number of ICM cells (Fig. 4).

The increase of ICM cells at the blastocyst stage was also seen at later stages in blastocyst outgrowths. Because the injected embryos did not express βGal after the blastocyst stage (data not shown), we transfected 4-cell embryos by incubation with Lipofectin for 8 hours; this procedure permitted analysis after the blastocyst stage. Embryo survival was low after this incubation. In two separate experiments, 12 of 200 parthenote embryos and 23 of 180 normal embryos survived to the blastocyst stage. Of the surviving blastocysts, 72% were positive for β-galactosidase activity. 4-cell embryos transfected with the Igf-1r and βGal plasmids were cultured with or without Igf-2 to the blastocyst stage. Embryos that survived to the blastocyst stage were placed in individual drops of medium containing serum in the presence or absence of Igf-2 and cultured for 3 days. Representative photomicrographs are shown in Fig. 5A-D. All parthenote embryos transfected with Igf-1r and treated with Igf-2 had morphologically identifiable ICM populations, whereas none of the untreated transfected embryos did. To quantify this difference, we measured the area of the ICM population and compared the treatment groups (Fig. 5E). The ICM population of the Igf-1r-transfected parthenotes treated with Igf-2 was significantly greater than that of the embryos not treated with Igf-2.

We also attempted to increase the ICM population of the parthenotes by culturing blastocysts in ES/LIF medium, which more closely mimics the maternal environment. ES/LIF medium is used to obtain and culture undifferentiated embryonic stem cells, which are derived from ICMs (Robertson, 1987). This medium contains serum and the maternal factor LIF, which promotes the growth of undifferentiated cells (Smith et al., 1992; Pesce et al., 1993). The blastocysts were cultured in ES/LIF medium on top of a layer of mouse SLN fibroblasts, which secrete LIF and other factors. This method resulted in a large increase in the ICM population from both normal and parthenote blastocysts (compare Fig. 6A,E with Fig. 6B,F). This effect was specific for these culture conditions, and was not seen with all mitogens. T-extra medium, which contains insulin and Ultrasr, a serum substitute, did not increase the parthenote ICM population (Fig. 6A,E), and, as shown above, did not maintain the parthenote ICM population past day 3. These results show that the mitogens and other factors contained in ES/LIF medium and secreted by SLN fibroblasts, but not insulin and Ultrasr alone, will maintain the parthenote ICM population, correcting one of the developmental defects of the parthenotes.

Mitogen-treated parthenotes differentiate predominantly into PE

We next addressed the question of whether maintenance of the ICM population would rescue the second defect of the parthenote ICMs, the preferential differentiation into PE. We increased the size of the ICM population by forming aggregations of two 8-cell embryos, which develop into one large blastocyst. The ICMs from these aggregates were then cultured for 3 days in T-extra medium, on a FN substrate. The parthenote ICMs again differentiated predominantly into PE cells, whereas normal ICMs formed a variety of cell types (data not shown).

We also used the protocol described above for ES/LIF medium to increase the size of the ICM. We isolated the ICMs after 4 days and placed them in T-extra medium on a substrate of FN, which promotes differentiation (Sutherland et al., 1993). Immunocytochemical analysis with anti-LN antibodies showed that, as before, the parthenote stem cell colonies differentiated predominantly into PE, whereas the normal stem cell colonies contained a variety of cell types, including PE, visceral endoderm-like cells, and ICM cells (Fig. 6C,D,G-I). These results suggest that even when the ICM cells are maintained initially, parthenotes still differentiate preferentially into PE cells.

DISCUSSION

Our results suggest that parthenotes are defective in two aspects of development: maintenance of the ICM population and differentiation of the ICM population. We found that the ICM population was maintained when the parthenotes were supplied with mitogenic agents, in the form of ES/LIF medium or the Igf-1r/Igf-2 pathway, suggesting that parthenote ICMs are not maintained because they are not proliferating.
Parthenote ICMs are not maintained and differentiate mainly into PE.

After 3 days in culture, parthenote ICMs had differentiated mainly into PE cells, whereas normal ICMs had differentiated into PE cells, visceral endoderm-like cells, and other cell types, and had maintained an ICM population. The maintenance of the ICM population by mitogenic factors suggests that parthenotes misregulate some component of the proliferation pathway. However, this does not explain why most of the differentiated cells were PE. There are two models that may explain the high proportion of PE cells. First, parthenotes may have a defect that pushes ICM cells to differentiate into PE. In normal ICM outgrowths, PE formation can be affected by a variety of factors, including the composition of the extracellu-

Fig. 3. Parthenote blastocysts express less Igf-2 and Igf-1r than normal blastocysts. Whole-mount in situ hybridization was performed on normal (left) and parthenote (right) blastocysts with an anti-sense probe for Igf-2 (top row), Igf-1r (middle row), and Insr (bottom row). Bar indicates 50 μm.
lar matrix (Behrendtsen et al., 1994), growth factors such as fibroblast growth factor-4 (Rappolee et al., 1994), and factors produced by the trophoblast, such as parathyroid hormone-related peptide (van de Stolpe et al., 1993; Behrendtsen et al., 1994). One or more of these factors may be altered in parthenotes. Another possible model is that the pathways leading to cell types other than PE are affected in such a way that these cells are not formed, or undergo apoptosis. We examined the apoptosis of normal and parthenote cultures and did not see any difference. At day 2 in culture, parthenotes had about 50% PE cells and 50% other cell types. Thus, it appears that other cell types are formed. However, classification of cells at this early stage is difficult, because they have not taken on their full differentiated state. Some of the non-PE cells in day 2 cultures were very close to the ICM and may not have had time to migrate away from the ICM and, thus, be classified as PE by morphology or by staining. In normal cultures, the proportion of total cells to PE cells did not change from day 2 to day 3; thus, at day 2, at least some non-PE cell types in normal cultures were truly non-PE, and not PE precursor cells. However, in parthenote cultures, nearly all the cells were PE at day 3, suggesting that most of the other cell types at day 2 were immature PE cells. This supports the hypothesis that non-PE cell types are not formed in parthenotes.

The differentiation of most of the parthenote stem cells and their lack of proliferation may be related. In many systems, there is an inverse relationship between differentiation and proliferation. For example, the myogenic transcription factor MyoD is sufficient to cause 10T1/2 cells to differentiate into muscle in vitro, and overexpression of MyoD leads to cell cycle arrest (reviewed by Olson, 1992). Thus, parthenote ICM cells may differentiate rather than proliferate. When we added agents that induce proliferation, we saw a decrease in differentiation. Moreover, treatment with ES/LIF or Igf-1r/Igf-2 led to larger ICMs. Removal of these agents caused the ICMs to resume their differentiation. However, as was the case for the smaller ICMs, the parthenote ICM cells again differentiated into PE cells, not other cell types. Thus, parthenotes have two developmental problems, one of ICM maintenance and another of ICM differentiation into a disproportionate amount of PE.

**Maternal repression of Igf-1r and Igf-2**

Using whole-mount in situ hybridization on blastocysts, we detected Igf-2 and Igf-1r in normal blastocysts. Much less Igf-
2 and Igf-1r was detected in parthenote blastocysts. Earlier work from this laboratory reported that Igf-2 mRNA is not detectable in parthenote blastocysts by reverse transcription-polymerase chain reaction (RT-PCR) (Rappolee et al., 1992). Recently, another group detected low levels of Igf-2 mRNA in parthenote blastocysts by using RT-PCR (Latham et al., 1994). Although it is possible that the occasional parthenote blastocyst expresses Igf-2, or that Igf-2 is expressed at a very low level in all parthenote blastocysts, our data suggest that parthenotes misregulate these two genes.

The diminished level of Igf-1r in parthenotes is interesting, because no evidence for gametic imprinting of this gene has been found, either in the targeted disruption of Igf-1r or in interspecies hybrids by using an mRNA phenotyping approach (Villar and Pedersen, 1994). It is possible that the occasional parthenote blastocyst expresses Igf-2, or that Igf-2 is expressed at a very low level in all parthenote blastocysts, our data suggest that parthenotes misregulate these two genes.

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Proliferation signals in vivo

We have shown that the ICM population is not maintained in parthenote embryos in vitro. However, parthenote embryos occasionally develop to the limb bud stage in vivo, overcoming the lack of ICM maintenance. Mice homozygous for the deletion of Igf-1r/Igf-2 develop to term (Baker et al., 1993; Liu et al., 1993). What is causing the ICM to proliferate in these cases? Trophoblast, supplied to parthenotes either as trophoblastic vesicles or by forming chimeras with tetraploid embryos, can rescue the stem cell defects of parthenotes developing in vivo (Allen et al., 1994; Barton et al., 1985). This suggests that maternal factors, available because of the invading trophoblast, are important in maintaining the proliferation of ICM cells. The maternal uterine epithelium secretes many factors that may affect the embryo, such as LIF and activin. Both have been shown to increase the proliferation of ES cells in culture (Heath and Smith, 1988; Nichols et al., 1990). Indeed, we have shown that an enriched culture medium containing LIF stimulates the production of ICM cells in parthenotes, and medium containing LIF has been used to generate parthenote ES cell lines (Allen et al., 1994). These maternal factors may cause proliferation of ICMs of mice lacking Igf-2/Igf-1r. The amount of trophoblast produced by the parthenote may give poor access to these factors, allowing only a small proportion of parthenotes to develop past the peri-implantation stage and overcome the ICM defects that we have characterized.

This study has allowed us to elucidate the nature of the two early developmental defects in parthenogenetic embryos. Our results point to the significance of communication from maternal tissues to embryo that can overcome these defects. Separate consideration of embryonic and maternal contributions in culture models such as the one used in this study may also be useful in dissecting early embryonic defects in other types of abnormal and mutant embryos.

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