Peri-implantation Mouse Development: Lessons from Genomic Imprinting

by

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Dedication

This thesis is dedicated to my parents, Hal and Merylyn Newman, who always encouraged me in whatever I was pursuing, to the rest of my family, especilly Colleen, who rescued me numerous times, and to Bill, for his emotional and scientific support throughout my graduate years.

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Zena Werb

Abstract

Peri-implantation development in the mouse: lessons from genomic imprinting. Erin D. Newman-Smith

In a process termed genomic imprinting, some genes, such as the insulin-like growth factor (Igf)-2 gene, are expressed only when inherited from one parent. Imprinted genes are key regulators of development in the mouse. Parthenogenetic mouse embryos, which contain only maternal DNA, do not develop normally. By characterizing the development of parthenogenetic embryos using an in vitro culture system that mimics peri-implantation development, stem cell defects were found in both embryonic and trophoblastic lineages. In both lineages, parthenogenetic stem cells were not maintained, due to a lack of proliferation. When expression constructs for the signaling receptor Igf-1 receptor, and its ligand, Igf-2, which are shown not to be expressed in parthenote blastocysts, were injected into parthenotes, the embryonic stem cells were maintained. A maternal factor, leukemia inhibitory factor, also maintained the embryonic stem cells. However, the maintenance of the embryonic stem cells was not able to overcome the second defect characterized, the differentiation of the embryonic stem cells predominately into parietal endoderm. The trophoblast lineage of parthenotes also lacked stem cell maintenance. The trophoblast giant cells of parthenotes showed a decreased rate of endoreduplication, and an altered nuclear structure, suggesting that the small amount of trophoblast that is produced in parthenotes is not normal. These defects suggest key steps in peri-implantation development that are controlled by imprinted genes.

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Chapter 1: Genomic imprinting in early mouse embryos

by Erin Newman-Smith

According to mendallian genetics, heterozygous progeny display the phenotype of dominant alleles. Which parent the dominant allele originated from is not important. However, more than 30 years ago, Helen Crouse discovered that the parental origin of chromosomes in the homopteran scale insect, Sciara, is important (Crouse, 1960). Sciara embryos initially have 3 copies of the X chromosome. At the eight cell stage, the embryo inactivates or eliminates one or both of the paternally derived X chromosomes. The maternally derived X chromosomes is always retained. She deduced that the chromosomes must be "imprinted" with parental information, such that the embryo knows which chromosomes are of maternal and which are of paternal origin. In mammals, it has been also known for some time that the two parental genomes are not equivalent and must be imprinted. Mouse embryos containing biparental, diploid amount of only maternal or only paternal chromosomes are not viable (Surani et al., 1990). The lethality is due to the mis-expression of genes which are dependent on the parental origin.

In mammals, certain regions of chromosomes are imprinted, rather than entire sets of chromosomes. These regions were initially determined by Cattanach and Kirk using mice with Robertsonian translocations, which when crossed can produce chromosomally balanced offspring with regions of parental disomy (Cattanach and Kirk, 1985). The offspring were analyzed for phenotypes that were dependent on the parent of origin of the disomy. They found 9 such regions that gave a phenotype if inherited from only one parent. Some of these regions only gave a phenotype if maternally disomic, some only if paternally disomic, and some either way. To date, 11 imprinted genes have been identified, some of which have been mapped, and are clustered within the regions identified by Cattanach and Beechey (Bartolomei, 1994). This suggests that chromosomal regions are regulated by imprinting. These regions also display parental allele specific DNA replication timing, such that one parental allele is replicated before the other. Non-imprinted regions show almost synchronous DNA replication (Kitsberg et al., 1993).

However, not all genes within these regions are imprinted, nor are all imprinted genes expressed from the same parental allele. For example, two imprinted genes, H-19 and Igf-2 are in close proximity, yet H-19 is expressed exclusively from the maternal allele, while Igf-2 is expressed from the paternal allele (Bartolomei, 1994; DeChiara et al., 1991). H-ras-1 and Gabrb3 map near H-19 and Igf-2 yet they shown no parental specific expression pattern (Villar and Pedersen, 1994). The insulin gene, Ins-2, and the trophoblast specific gene, Mash-2, both of which are imprinted, also map to this region (Giddings et al., 1994; Guillemot et al., 1994). Another region occurs in the center of chromosome 7 in the mouse, where three imprinted genes have been identified. These genes, the small ribonucleoprotein N gene (Snrpn), and, in the human, within 250 Kb, PAR-1 and PAR-5 are all expressed from the paternal allele (Leff et al., 1992; Reed and Leff, 1994; Sutcliffe et al., 1994). On mouse chromosome 17, IGF-2 receptor (Igf-2r) and the protooncogene, mas, are both expressed from the maternal allele (Stoger et al., 1993; Villar and Pedersen, 1994). In between these is the gene for plasminogen, which is not imprinted (Stoger et al., 1993). Thus, while certain regions of the mouse chromosomes contain several imprinted genes, regulation by imprinting is gene specific.

Regulation by imprinting is also tissue specific and developmentally specific. For example, Igf-2 is imprinted in all tissues that have been tested in the adult except for two areas of the brain, the choroid plexus and the leptomenegis. Here, Igf-2 is expressed from both the maternal and the paternal alleles (DeChiara et al., 1991). Ins-2 is imprinted in the embryonic yolk sac, but not in the pancreas of the embryo proper (Giddings et al., 1994). Xist, a gene which regulates X inactivation, is also imprinted tissue specifically. The paternal X chromosome is preferentially inactivated in the extraembryonic tissues in the mouse, while X inactivation is random in the embryo proper (reviewed in (Monk and Grant, 1990). Some genes are regulated developmentally. For example, mas is expressed form both alleles until day 11, when it is expressed from only the paternal allele (Villar and

Pedersen, 1994). Therefore, while certain regions of chromosomes are subject to imprinting, this regulation is gene specific, tissue specific, and developmentally specific.

Mechanism of genomic imprinting

How imprinting acts to regulate gene expression is currently being investigated in many labs. Any mechanism to explain genomic imprinting must answer these questions, and meet certain criteria. There must be a modification of the DNA in some way that marks its parental origin. This imprint must be established before pronuclear fusion of the gametes. This modification must be heritable so that daughter cells can recognize the parental origin of its DNA. As gametes are produced, this mark must be removed such that maternal DNA inherited by a son can be passed on as paternal DNA. This mark must affect gene expression, the functional expression of genomic imprinting.

DNA methylation can meet these criteria (for review see (Barlow, 1993; Leighton et al., 1995; Razin and Cedar, 1991; Razin and Cedar, 1994). Methylation of the CpG dinucleotide is common in mammalian DNA. Differential DNA methylation based on the parental origin of the DNA could serve as the imprint. DNA methylation is stably passed from one cell to its daughter cells by methyltransferase, an enzyme that uses hemimethylated DNA as its substrate. Through several rounds of DNA replication without methyltransferase activity (or perhaps via an undiscovered demethylase), the DNA will become unmethylated. Thus methylation is erasable. Methylation can also affect gene transcription. CpGs are found in clusters, termed CpG islands, while GpCs are dispersed throughout the genome (Bird, 1986). CpG islands often fall on the promoter region of genes. When these CpGs are highly methylated, transcription is inhibited. Methylation of many transgenes prevents their transcription when transfected into tissue culture cells (Boyes and Bir, 1991; Murray and Grosveld, 1987). For example, a gamma globulin transgene that is methylated in its CpG island and then transfected into cells in vitro is not

transcribed, while the unmethylated transgene is (Busslinger et al., 1983). Thus, methylation can fulfill all the criteria of the mechanism of genomic imprinting. But, is it?

First, is there differential methylation of imprinted genes? In six of the endogenous imprinted genes, there is. For example, Igf-2r has two regions that are differentially methylated. One region is a CpG island around the promoter, and is hypermethylated in the paternal allele, which is not transcribed. The other region is in an intron, and it is methylated on the maternal, transcribed allele. The allele specific methylation pattern of the CpG island was not present in the male gametes, but probably set up in the early embryo. The allele specific methylation pattern of the intron, however, was present in eggs and not in sperm. Thus this region could function as an imprint, telling the cell which DNA is maternal (Stoger et al., 1993). Like Igf-2r, H-19, Igf-2 and Xist all have allele specific methyl moieties that are present in the gametes, and the unmethylated allele is maintained during a general wave of methylation that occurs after the blastocyst stage (Ariel et al., 1995; Ferguson-Smith et al., 1993; Kafri et al., 1992; Tremblay et al., 1995; Zuccotti and Monk, 1995). Thus, methylation could be the mark on the DNA that distinguishes maternal from paternal alleles.

Does the parental allele-specific methylation affect transcription of the imprinted genes? In mice that have a targeted deletion of the maintenance methyltransferase, H-19 and Igf-2 showed abnormal transcription (Li et al., 1992). H-19 was transcribed from both alleles, and Igf-2 was not transcribed. Therefore, methylation is in some aspect, important for imprinting. It could be important for initiating the allele-specific transcription patterns, or it could be important for maintaining the allele-specific transcription patterns. In X chromosome inactivation, methylation maintains the transcriptional off state. By examining expression of HPRT in early mouse embryos undergoing X-inactivation, Lock and Martin found that this gene is transcriptionally silent before it becomes methylated (Guillemot et al., 1994; Lock et al., 1987). Therefore, methylation cannot be initiating the transcriptional inhibition. Methylation of the gene's CpG island occurs at a later stage, and

this methylation maintains the transcriptional silence. Several lines of evidence suggests that methylation is also important for maintaining the differential transcription in imprinted genes as it is in X-inactivation. In H-19, Igf-2, Igf-2r and Xist the parental allele specific methylation that is set up before pronuclear fushion is not in the CpG islands where methylation has been shown to inhibit transcription (Ariel et al., 1995; Ferguson-Smith et al., 1993; Sasaki et al., 1992; Stoger et al., 1993; Tremblay et al., 1995; Zuccotti and Monk, 1995). In fact, in some cases (Igf-2r-Igf2), the methylated allele is the one that is expressed. In these cases, methylation is not inhibiting transcription. Methylation of the CpG islands occurs specifically in the alleles that are not expressed, and this occurs after fertilization. For example, the parental-allele specific methylation pattern that is set up before pronuclear fushion in Igf-2r is in an intron. The maternal allele is methylated and it is this allele that is expressed. Methylation of the CpG island occurs later in the paternal allele, maintaining this allele in the off state (Stoger et al., 1993). H-19 also has two regions that are methylated differently depending on the parental origin of the allele. One region is within the gene, and it is set up before pronuclear fushion. The methylated allele, in this case, is not transcribed. Another region is within the CpG island which becomes methylated after fertilization in the allele that is not transcribed (Ferguson-Smith et al., 1993; Tremblay et al., 1995). Thus, as in X-inactivation, methylation plays a role in maintaining the transcription silence of one of the alleles.

If methylation is not initially inhibiting transcription, how does the differential methylation lead to differential transcription? Methylation may cover sites of transcription factors either preventing their binding, or perhaps facilitating their binding. In the case of the Igf-2r, it has been suggested that methylation of the intron region prevents a transcriptional regulator from binding (Stoger et al., 1993). This leads to the methylated allele being transcribed. Alternatively, methylation may affect the transcription of one gene that is the regulator for the entire chromosomal imprinted region. An example of a gene that regulates other genes in its vicinity is the imprinted Xist gene which may cause X

chromosome inactivation. In both mouse and human, its expression precedes X inactivation, and maps to the X-inactivation center (a locus determined genetically to be causal in X inactivation), and thus controls the transcription of genes on the inactive X chromosome. Xist is a large mRNA that has no evolutionary conserved open reading frame, is not associated with the translational machinery, and has been shown by in situ hybridization to localize to the inactive X chromosome, suggesting that it functions as a RNA or as a chromosome localizing center (Brockdorff et al., 1992; Brown et al., 1992). Other imprinted genes may function like Xist to control transcription of other genes. This may be true for H9 and Igf-2 as well as the Prader-Willi/Angelman's locus. As discussed above, imprinted genes are clustered on the chromosomes into regions, such as the distal tip of chromosome 7 that contains H-19 and Igf-2. H-19 and Igf-2 are very close to each other (90 kb), yet are imprinted in opposite ways. Deletion of the H-19 gene (and several kb 5' to it) result in the expression of Igf-2 from both alleles, instead of just the paternal allele (Leighton et al., 1995). Another imprinted gene, Ins-2, is much farther away, and is also expressed from both alleles when H-19 is deleted. Thus, H-19, or some elements in the 5' region, seems to control imprinting of the entire region. Like Xist, H-19 probably functions as an mRNA as it has no evolutionarily conserved open reading frame, and is not associated with ribosomes (Brannan et al., 1990). Another region that is regulated in this manner is the Prader-Willi locus (Sutcliffe et al., 1994). Three imprinted genes have been mapped to this region in the human on chromosome 15 (syntenic to mouse chromosome 7); Snrpn, PAR-1 and PAR-5. A deletion 5' of Snrpn not only abolishes the transcription of Snrpn, but also abolishes the transcription of PAR3 and PAR5 (which are 300 kb and 100 kb away respectively). Thus the transcription of these genes is controlled by a single element. Like Xist and H-19, PAR3 and PAR5 have no obvious open reading frame, and may function as mRNAs. Thus in general, genes in imprinted regions may be controlled by one element that is sensitive to the allele-specific DNA methylation differences. This suggests that the allele-specific methylation differences may be in fact, initiating the

differential transcription, but not by directly inhibiting transcription. The allele-specific methylation patterns that are set up before pronuclear fushion may initiate differential transcription of all the imprinted genes in one region by covering binding sites for transcription factors of a controlling gene, such as Xist or H9.

Biological consequences of genomic imprinting

What is the consequence of genomic imprinting for the organism? Genes regulated by genomic imprinting are required for normal cellular function. Cases of parental disomy or hemizygosity can lead to diseases, including cancers, in humans. For example, Prader-Willi syndrome and Angelman's syndrome are caused by the parental specific chromosomal deletion. Prader-Willi syndrome is characterized by failure to thrive in infancy, mental retardation, obesity, hypogonadism and small hands and feet. Deletions of this locus that are inherited from the father result in the syndrome, while offspring that inherit deletions from their mother are unaffected. The three imprinted genes found in this region ,Snrpn, PAR-5 and PAR-1, may contribute to the disease (Leff et al., 1992; Reed and Leff, 1994; Sutcliffe et al., 1994). All are expressed solely from the paternal allele. In this same chromosome region (about 400 kb away), another deletion causes Angelman's syndrome. This syndrome is characterized by mental retardation, seizures, hyperactivity and bouts of inappropriate laughter. Offspring that inherit this deletion from the father are unaffected, while offspring that inherit this deletion from the mother have the disease. Other human disease also show an imprinted component. Maternal inheritance of myotonic dystrophy leads to a severe congenital form of the disease while paternal inheritance leads to a less severe adult onset form (Howeler et al., 1989). Imprinting may also be involved in Huntington's disease where paternal inheritance leads to earlier onset of the disease than maternal inheritance (Ridley et al., 1992).

Several cancers are also caused by parental specific chromosomal deletions or duplications. Imprinted genes in these regions are then not expressed at the same levels as

in normal cells, and this, presumably leads to cancer. In some cancers, the tumor cells have become hemizygous for certain chromosomal regions. In sporadic cases of Wilms' tumor, for instance, a loss of heterozygosity is seen, where the paternal allele is preferentially maintained. Thus, an imprinted gene on this chromosome may be responsible for tumor growth. Indeed, Jinno et al found that the Wilms' tumor suppresser gene (WT1) was imprinted, and was lost in sporadic cases of Wilms' tumor (Jinno et al., 1994). Rhabdomyosarcoma, bilateral retinoblastoma and osteosarcoma also show a loss of heterozygosity. In these cancers, it is also the paternal allele that is maintained (reviewed in (Reik and Surani, 1989). By analyzing these cancers, it may be possible to determine which imprinted genes are responsible, what the function of these genes is, and how this leads to tumor growth.

As well as being required for normal cellular function in the adult, genes regulated by genomic imprinting are also required for normal development of the embryo. Mouse embryos containing only maternal DNA (parthenotes, which are uniparental or gynegenotes which are biparental) or mouse embryos containing only paternal DNA (androgenotes) do not survive past early post-implantation. (reviewed by (Surani et al., 1990). In parthenotes, embryos developing in vivo often form masses of parietal endoderm cells (Sturm et al., 1994). Occasionally they progress to the early limb bud stage as morphologically normal, but small embryos, with very little trophoblast (Kaufman et al., 1977; Sturm et al., 1994) Androgenetic embryos at best, form large amounts of trophoblast, but have poor embryonic development (Surani et al., 1984). Genes that are imprinted, and thus expressed at aberrant levels in disomic embryos are presumably responsible for the phenotypes observed.

Determining how these embryos fail may help us understand the function of imprinted genes in early development. By characterizing how parthenotes develop in vivo, I have determined that the parthenotes are missing proliferation signals, specifically in stem cells, of both the embryonic and trophoblast lineage. Differentiation pathways are also

abnormal in parthenotes. I have tested the hypothesis that genes not expressed in parthenotes, but expressed in normal embryos, may counter the proliferation and differentiation defects in the parthenotes.

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Chapter 2:
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.

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 imprinted have a wide range of functions, from splicing factors, such as *Snrpn* 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., submitted for publication) 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 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 mineral oil (Sigma) at 37°C with 5% CO_2 in air. Parthenotes were activated essentially as described by Kaufman (1978). Briefly, 1-cell embryos were isolated from superovulated B6CBA females and activated with 6% ethanol. The oocytes were then cultured for 5 hours in 1 mg/ml cytochalasin D to prevent extrusion of the second polar body. The embryos were washed extensively in FM-I (Spindle, 1980) and cultured as described for the normal embryos.

Culture conditions for ICMs

Embryos were cultured for 4-5 days to the expanded/hatching blastocyst stage, then overnight in T-extra medium (T+2XAA [Spindle, 1990] supplemented with insulin, Ultraser (IBF Biotechnics, France), Mito+ (Collaborative Research), sodium pyruvate, and calcium lactate [Sutherland et al., 1993]). Immunosurgery was performed essentially as described (Solter and Knowles, 1975). Embryos were incubated with a 1:5 dilution of rabbit anti-mouse antibody for 10 minutes at 37° C, washed, then incubated in rat serum (Harlan Bioproducts, 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.

In experiments designed to increase the ICM cell population, blastocysts were cultured for 3 days on SLN fibroblasts in embryonic stem (ES) cell medium supplemented with leukemia inhibitory factor (LIF) (ES/LIF medium). SLN fibroblasts are mouse fibroblasts stably transfected with a *neo*^{r} expression vector and with a LIF expression vector (generous gift of Elizabeth Robertson, Harvard University, Cambridge, MA). ES/LIF medium consists of DME H-21 supplemented with 15% fetal bovine serum, nucleosides, nonessential amino acids, mercaptoethanol (see Robertson, 1987, for specific concentrations) and supplemented with 5% medium conditioned by Chinese hamster ovary (CHO) cells containing a gene for LIF (CHO-LIF cells; gift of Genetics Institute, Cambridge, MA). After 3 days in ES/LIF medium, ICMs were isolated and placed in 5-μl drops of T-extra medium on a substrate of 50 μg/ml FN.

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

Apoptotic cells in day 2 ICM cultures were labeled with digoxigenin-dUTP by using terminal deoxynucleotidyl transferase exactly as described in the ApopTag 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 pre-implantation 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) (Burdsal 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 ImaGene 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 *Insr* 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).

Transfection protocol

Preimplantation embryos were transfected essentially as described by Cross et al. (1995). Pre-compaction 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 (Behrendtsen et al., 1994). After 3 days in culture, three cell types could be identified by morphological and molecular criteria: PE, 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 α-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 only 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., 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 pre-implantation 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 specificty 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. Four-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. 5 A-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 parthenote cultures 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 treatment 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 Ultraser, 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 Ultraser 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 extracellular 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 a regulator of *Igf-1r* expression may be imprinted, or that *Igf-1r* expression is diminished in parthenotes for reasons other than gametic imprinting.

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 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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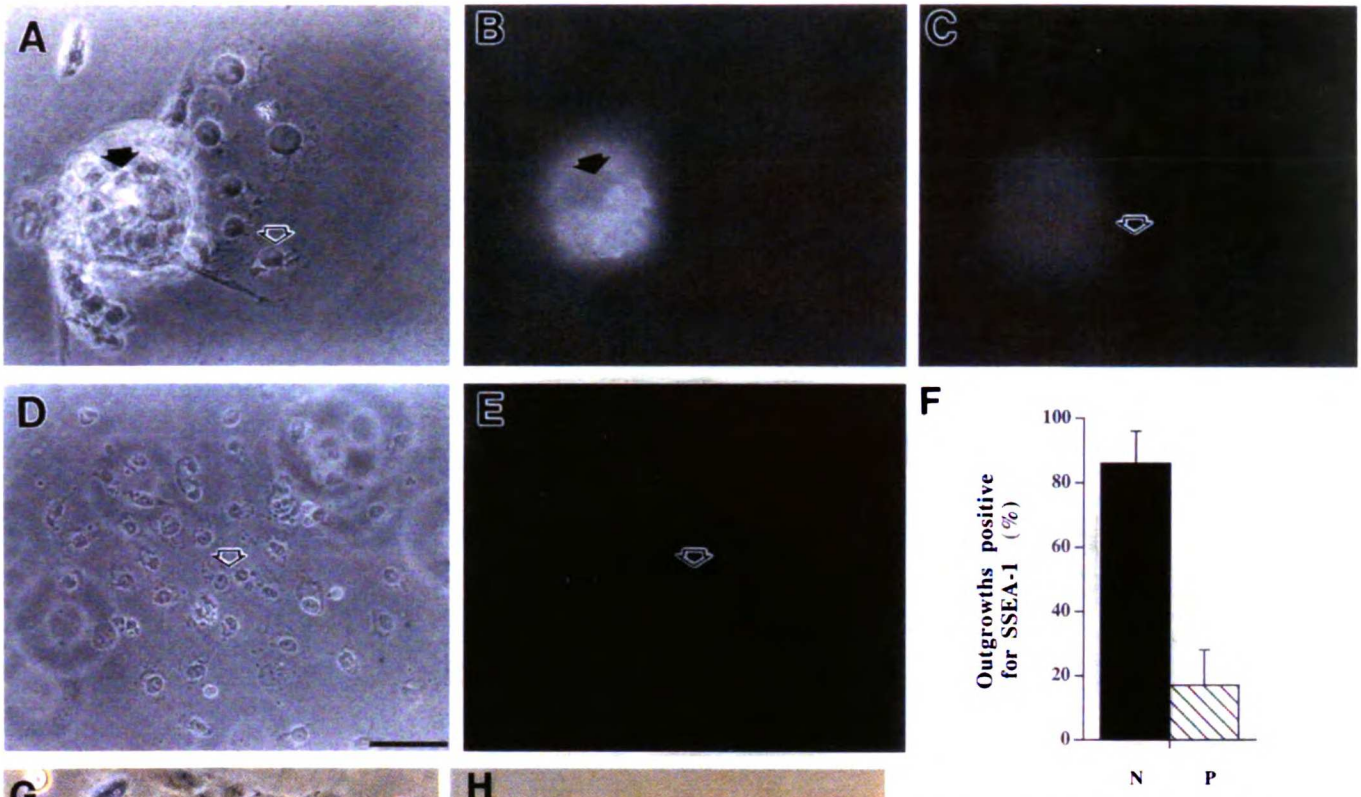


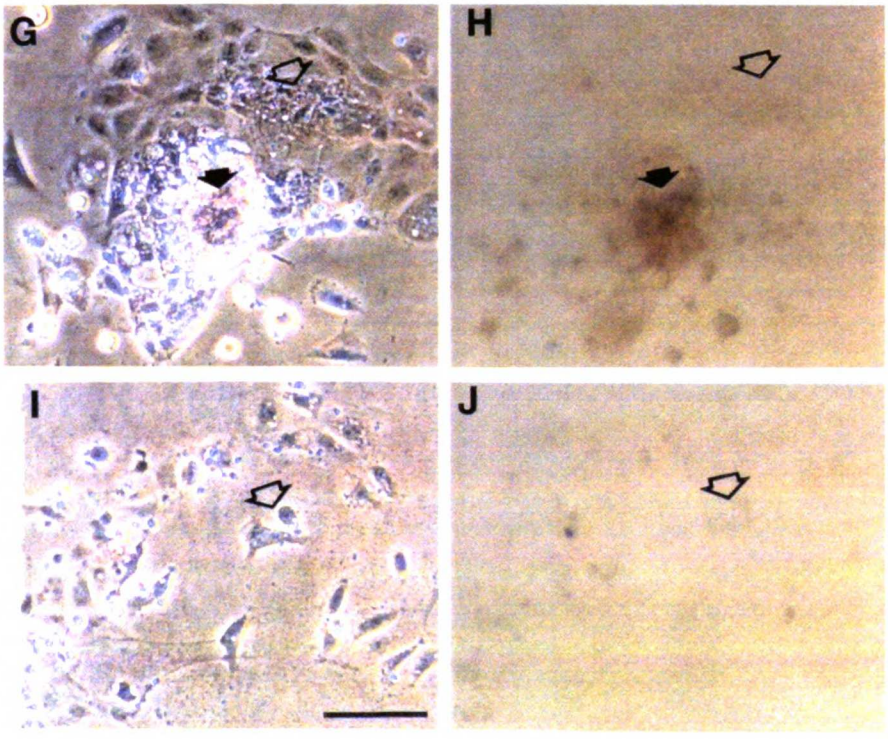


Fig. 1. Parthenote ICM cultures do not mainta an undifferentiated stem cell population. Normal (A-C) and parthenote (D,E) ICM outgrowths were cultured for 3 days and staine with anti-SSEA-1 antibody, a marker for undifferentiated stem cells. (A,D) Phase-contrast photomicrographs; (B,C,E) fluorescer photomicrographs. A and C are the same plan of focus, and B is a plane of focus through the ICM. Only ICM cells were positive for SSEA- (filled arrow). Cells with PE morphology were not positive for SSEA-1 (open arrows). (F) More normal ICM outgrowths (solid bar) had or more cells positive for SSEA-1 than did parthenote ICM outgrowths (striped bar). Normal, 76±10%, n=17; parthenote, 17±11%, n=17; P<0.001. Values are expressed as mean ± s.e.m; Student's t-test was used to determine P values. In situ hybridization with a probe for *Rex-1* was performed on normal (G,H) and parthenote (I,J) ICM outgrowths after 3 days i culture. (G,I) Phase-contrast photomicrograph and (H,J) bright-field photomicrographs. Fille arrows indicate cells expressing *Rex-1* and ope arrows indicate nonexpressing PE cells. Bars indicate 50 μm.

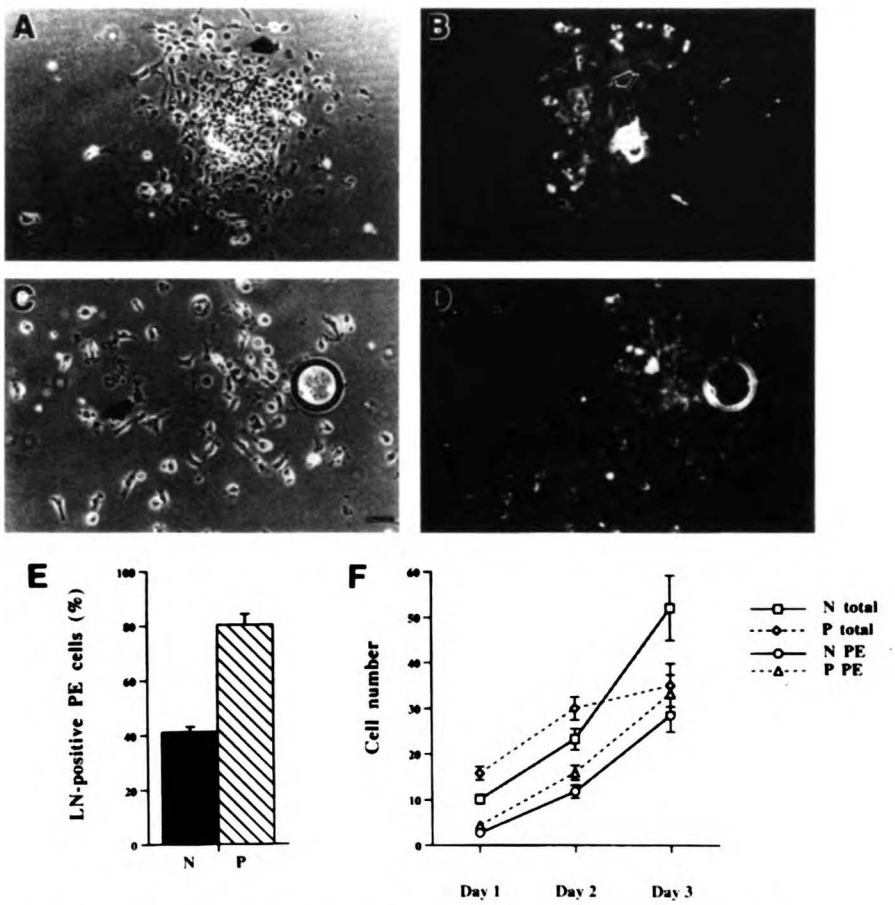


Fig. 2. Parthenote ICMs differentiate predominantly into PE. Normal (A,B) and parthenote (C,D) ICM outgrowths were stained with anti-LN antibody. (A,C) Phase-contrast photomicrographs; (B,D) fluorescent photomicrographs. Filled arrows point to PE cells; open arrows point to non-PE cells. Bar indicates 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.

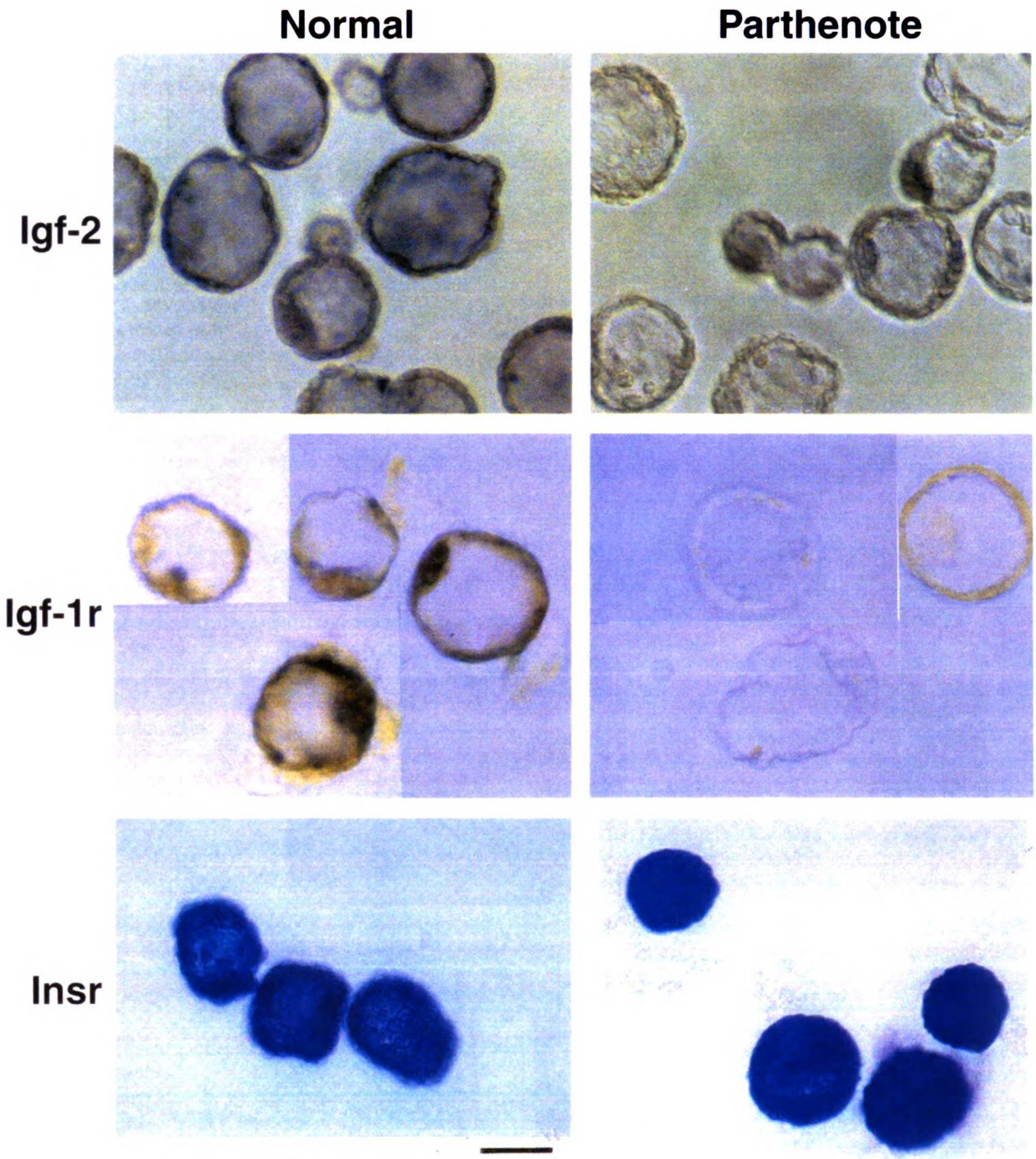


Fig. 3. Parthenote blastocysts express less *Igf-2* and *Igf-1r* than normal blastocysts. Whole-mount in situ hybridization was performed on normal (left) and parthenote (right) blastocysts with an anti-sense probe for *Igf-2* (top row), *Igf-1r* (middle row), and *Insr* (bottom row). indicates 50 μm.

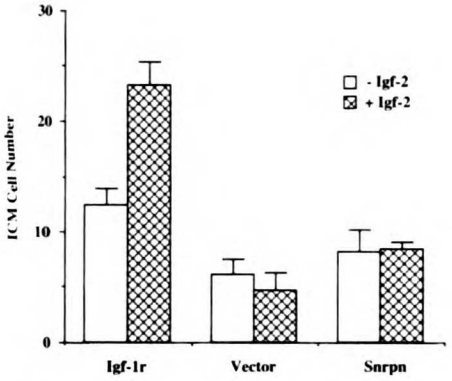


Fig. 4. *Igf-1r* and *Igf-2* increase the number of ICM cells in parthenote blastocysts. Parthenote embryos injected at the 2-cell stage with *Igf-1r* and treated with Igf-2 (open bars) had more ICM cells at the blastocyst stage (23.21±2.04, *n*=24) than did embryos injected with *Igf-1r* but not treated with Igf-2 (cross-hatched bars) (12.4±1.5, *n*=10, *P*=0.003). ICM cell number was not significantly altered by injection with the expression vector alone, either with Igf-2 (6.1±1.31, *n*=9) or without Igf-2 (4.7±1.5, *n*=11), or with the imprinted gene *Snrpn*, either with Igf-2 (8.23±1.09, *n*=13) or without Igf-2 (8.45±0.6, *n*=11).

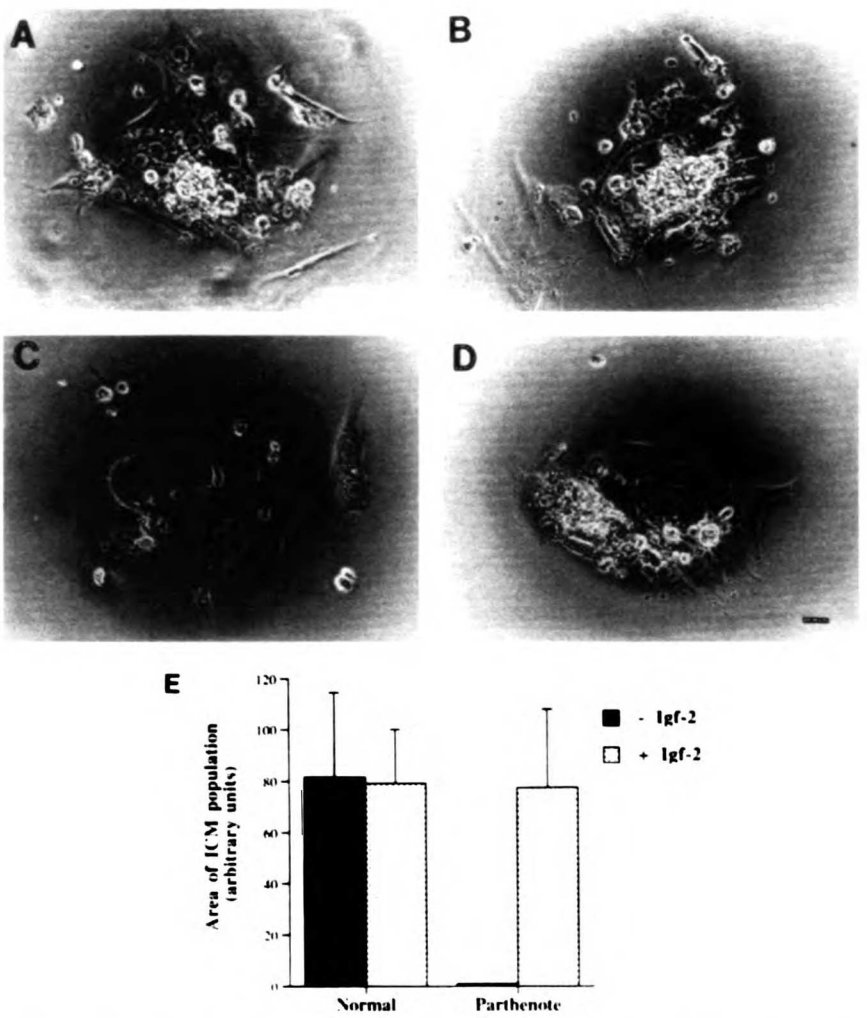
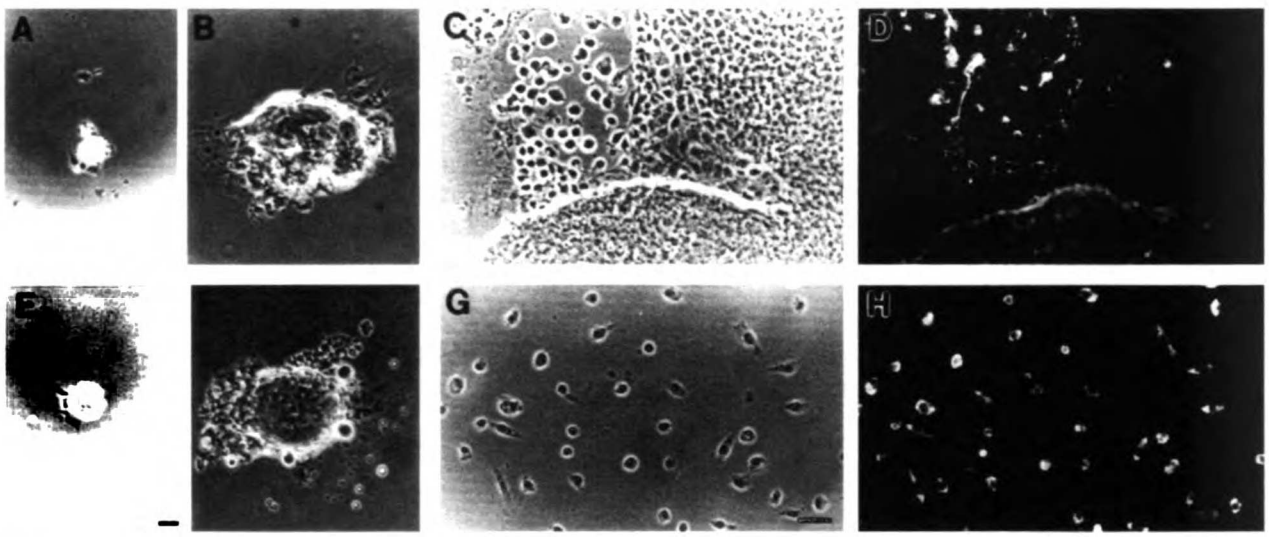


Fig. 5. *Igf-1r* and *Igf-2* maintain the ICM population in parthenotes. Normal (A,B) and parthenote (C,D) embryos were transfected with *Igf-1r* and cultured without Igf-2 (A,C) or with Igf-2 (B,D). Representative phase-contrast photomicrographs are shown. Arrows point to the ICM population. Bar indicates 50 μm. (E) The area of the ICM population was identified by using morphological criteria and measured in arbitrary units. No difference was detected between normal (N) embryos transfected with *Igf-1r* and cultured without Igf-2 (solid bar, 81.5±33.0 units, *n*=10) or with Igf-2 (stippled bar, 79.0±21.0 units, *n*=13). The undifferentiated stem cell population of parthenotes (P) transfected with *Igf-1r* and cultured with Igf-2 was significantly greater (77.3±30.5, *n*=6, *P*=0.03) than that of transfected parthenotes cultured without Igf-2 (0±0, *n*=6).



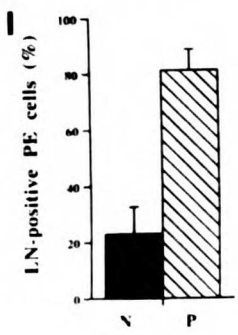


Fig. 6. Mitogen-treated parthenotes increase their ICM population, but still differentiate predominantly PE. (A-D) Normal embryos; (E-H) parthenotes. (A,E) Phase-contrast photomicrographs of blastocyst I cultured for 1 day in T-extra medium. (B,F) Phase-contrast photomicrographs (same magnification as A of ICMs isolated from blastocysts cultured in ES/LIF medium for 5 days and then in T-extra medium f day. (C,D,G,H) ICMs from blastocysts grown in ES/LIF medium were isolated and differentiated for 3 in T-extra medium and stained with anti-LN antibody. (C,G) Phase-contrast photomicrographs; (D,H) fluorescent photomicrographs. Bar represents 50 μm. (I) The proportion of PE cells was determi in cultures of ICMs from blastocysts grown in ES/LIF medium and differentiated for 3 days in T-extra medium. Parthenote ICMs (striped bar) had a higher percentage of total cells that were PE than did nor ICM cultures (solid bar). Normal, 22.9±9.7% (n=7); parthenote, 81.1±7.6% (n=19; P<0.001).

Chapter 3: Functional analysis of trophoblast giant cells in parthenogenetic mouse embryos

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ABSTRACT

In the mouse, diploid embryos containing only maternal DNA (parthenotes) fail, in part, because the trophoblast is not induced to grow by the inner cell mass (ICM) of the embryo. In this study, we have determined whether any of the defects in parthenotes may arise from alterations in trophoblast function . We examined the expression of genes important for trophoblast function and found several trophoblast genes were normal in the parthenotes: E-cadherin, a cell adhesion molecule, was expressed normally in both the ICM and trophectoderm of parthenote blastocysts and blastocyst outgrowths; the gene for Hxt, a basic helix-loop-helix factor that regulates trophoblast development, was expressed in both zygotic and parthenogenetic giant cells; placental lactogen -1 (mPL-1), a hormone that is normally secreted by giant trophoblast cells, was expressed in most of the parthenote trophoblast cells, as it was in most of the normal trophoblast cells; and the 92 kDa matrix metalloproteinase, gelatinase B (MMP-9), was secreted by both zygotic and parthenogenetic blastocyst outgrowths. However, we noted that a subpopulation of trophoblast cells in parthenogenetic embryos had decreased DNA replication and significantly fewer nucleoli per nucleus than zygotic embryos. Moreover, the parthenogenetic trophoblast cells had a decreased viability in blastocyst outgrowths in culture. These data suggest that, while parthenote embryos are able to initiate trophoblast differentiation, the stability and continued differentiation of trophoblast giant cells may be abnormal. Thus, the deficiency of a subset of trophoblast giant cells may contribute to the decline of parthenogenetic embryos.

INTRODUCTION

Normal development in mammals requires that a full set of chromosomes be contributed to the offspring by each parent, because the expression of certain genes is dependent on the gamete, sperm or egg, from which the gene is inherited. This type of gene regulation is termed genomic imprinting because the gene must be marked, or imprinted with gamete of origin information. For example, in the mouse, the paternal allele of insulin-like growth factor (Igf) -2 is expressed, but the maternal allele is silent in most tissues(DeChiara *et al.*, 1991).

Embryos that are either completely or partially disomic fail in development, presumably because essential genes are imprinted, and thus, mis-expressed in these embryos. Disomic embryos develop in a characteristic fashion, with embryos containing only maternal DNA (parthenotes or gynogenotes) developing in a complementary fashion to embryos containing only paternal DNA (androgenotes) (Barton *et al.*, 1984; Surani *et al.*, 1984). Occasionally, parthenotes and gynogenotes can develop to day 12 as normal appearing, but small embryos, with little trophoblast (Kaufman *et al.*, 1977; Sturm *et al.*, 1994; Varmuza *et al.*, 1993). On the other hand, androgenotes that survive to day 12 have unorganized embryos with significant amounts of trophoblast (Barton *et al.*, 1984; Surani *et al.*, 1984). This suggests that trophoblast growth is in some way controlled by genes that are regulated by genomic imprinting.

In the early mouse embryo, trophoblast growth is tightly controlled by the inner cell mass (ICM), the portion of the mouse blastocyst that will form the embryo proper (reviewed by (Cross *et al.*, 1994; Ilgren, 1983). Removal of the ICM by microsurgery (Gardner, 1972) or by irradiation (Goldstein *et al.*, 1975) causes the remaining trophectoderm to cease dividing and to differentiate. Reintroduction of an ICM shortly after microsurgery leads to normal trophectodermal proliferation (Gardner *et al.*, 1973). At the late blastocyst stage, trophectoderm cells that are not in contact with the ICM, the mural trophectoderm, cease to

divide and their nuclei enlarge, presumably becoming giant cells, containing chromosome complements of more than 4 N. These cells form the primary trophoblast giant cells that secrete hormones and proteinases important for normal function. The cells that are in contact with the ICM, the polar trophectoderm cells, continue to divide, and remain diploid, giving rise to mural trophectoderm, the extraembryonic ectoderm which overlays the embryo proper, and the ectoplacental cone (EPC). Cells within the EPC continue to divide, and provide the stem cells for the trophoblast population. Cells at the periphery of the EPC, no longer in close contact with the ICM, cease to divide, and differentiate into secondary giant cells. Combined, these tissues form the embryonic contribution to the placenta.

In parthenogenetic embryos, trophoblast development is impaired. The trophectoderm does not proliferate when near the ICM (Barton *et al.*, 1985). Chimeric embryos of parthenote ICMs placed inside zygotic trophoblast vesicles are capable of inducing the trophectoderm to proliferate in vivo. However, chimeric embryos consisting of normal ICMs placed inside parthenote trophoblast vesicles do not maintain proliferation of parthenote trophectoderm (Barton *et al.*, 1985). These embryos often develop as poorly as parthenogenetic embryos. These data suggest that there is a cell-autonomous defect in the parthenote trophectoderm that prevents it from responding to the normal ICM. However, except for the failure to respond to ICM signals, the functional characteristics of the diminished amount of trophoblast that is produced by the parthenotes has not been elucidated. In this study, we have compared the functional properties of trophoblast from parthenogenetic embryos to that of normal zygotic embryos.

MATERIALS AND METHODS

C57BL/6JxCBA/J)F1 mice (B6CBA) were obtained from Jackson Laboratories (Bar Harbor, ME). Pregnant mares' serum gonadotropin was purchased from Sigma Chemical

co. (St. Louis, MO). Human chorionic gonadotropin (hCG) was obtained from Serono (Randolph, MA). Dulbecco's modified Eagle's medium (DMA) was prepared by the Cell Culture Facility (University of California, San Francisco). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Fluorescein-conjugated swine anti-rabbit immunoglobins were purchased from Dakopatts (Santa Barbara, CA). Rabbit antiserum to mouse placental lactogen (mPL-1) was kindly provided by Dr. Frank Talamantes (Colosi *et al.*, 1987). Rabbit antiserum to ID-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP) coupled antibodies to rabbit were purchased from Amersham. DAB was purchased from Sigma.

Embryo collection and parthenogenetic activation

Mice were housed in a pathogen-free mouse room on a standard 12 hour light/dark cycle, and embryos collected from superovulated females as described (Newman-Smith and Werb, 1995). The embryos were cultured in TE medium (Spindle, 1990) under mineral oil (Sigma) at 37 C with 5% CO^2 in air. Parthenotes were activated exactly as described (Newman-Smith and Werb, 1995). After the embryos had reached the expanded blastocyst stage, 3.5-4.5 days in culture, they were cultured in individual 5 µl drops of DME plus 10% FCS under oil.

Immunocytochemical analysis

Immunocytochemical analysis was performed essentially as described previously (Newman-Smith and Werb, 1995). Briefly, blastocyst outgrowths were fixed in freshly prepared, cold 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes. After thorough washing, the samples were permeabilized with 0.1% Triton in PBS with 1% bovine serum albumin (BSA). Samples were blocked in 10% FCS in DME before primary antibody was added at a 1:1000 dilution at RT for 1 hr. For mPL-1 immunocytochemistry, fluorescein-labeled swine anti-rabbit immunoglobin antibody was

used at a dilution of 1:1000. For immunocytochemistry with the anti-Id-1 antibody, HRP-labeled anti rabbit antibodies were used at a dilution of 1:1000. For detection of HRP, DAB was used following standard techniques.

Zymography

Blastocysts were grown in serum-free T-extra medium (Newman-Smith and Werb, 1995). Conditioned medium from blastocyst outgrowth cultures were collected after 3 days and analyzed by gelatin zymography (Herron *et al.*, 1986b; Herron *et al.*, 1986a). Samples were electrophoresied on 10% polyacrylamide gels containing 1 mg/ml gelatin. Gels were washed twice in 2.5% Triton X-100 for 15 minutes each wash, and then incubated in 50mM Tris-Hcl, 10 mM CaCl2, pH 7.8. Gels were then stained in 0.5% Coomassie Blue R250, and destained in 50% methanol. Proteinases in the sample degrade the gelatin incorporated into the gel and are visualized as clear areas on a blue background.

BUDR incorporation

In these experiments, blastocysts were cultured for 3 days past the blastocyst stage. One µl of a concentrated, pre-warmed Brdur stock was added to the cultures for a final concentration of 10 µM. After the indicated times (from 1 hr to 8 hrs), samples were fixed in cold (-20 °C) methanol for 20 minutes, and allowed to air dry. Immunocytochemistry using an anti-BUDR antibody was performed as described by the manufacturer. Briefly, samples were incubated in 2 N HCl at 37 °C for 30 min to denature the DNA, then washed extensively in PBS. Samples were incubated with anti-BUDR antiserum diluted 2:5 in 0.5% Tween-20 at ambient temperature for 30 min, followed by PBS washes, and incubation with biotin conjugated sheep anti-mouse IgG (Sigma) for 30 min at ambient temperature. The samples were then washed in PBS, incubated with fluorescein-conjugated streptavidin (Vector Laboratories), and examined using epifluorescence.

RESULTS

Parthenogenetic blastocysts develop fewer trophoblast giant cells than zygotic blastocysts

In vivo, parthenotes develop significantly less trophoblast than normal embryos (Barton *et al.*, 1985). We characterized the growth of parthenote and zygote blastocysts using an in vitro outgrowth assay (Behrendtsen *et al.*, 1995; Newman-Smith and Werb, 1995). Parthenote and zygotic blastocysts were placed individually in drops of culture medium containing serum and observed for up to 6 days. During this time in culture, the blastocyst hatches from the zona pellucida and attaches to the dish, and the trophoblast then spreads out, forming a layer surrounding the ICM. Representative photomicrographs of zygotic and parthenogenetic blastocysts after 5 days of outgrowth are shown in Fig. 1A. We distinguished trophoblast giant cells by the larger size of their nuclei in comparison to other cells. After 3 days of culture in the outgrowth assay, the parthenote and zygotic embryos had similar numbers of trophoblast giant cells (Fig. 1B). However, from day 3 to day 5, the zygotic cultures had increased numbers of giant cells, while the parthenotes did not, resulting in significantly more giant cells at day 5 in zygotic outgrowths than in parthenogenetic outgrowths. Thus, similar to observations in vivo, parthenotes in our culture system had significantly reduced numbers of trophoblast cells, suggesting that this in vitro system mimics in vivo development.

Parthenote giant cells express normal trophoblast differentiation markers

Our results suggest that parthenote blastocysts initially differentiate to give a normal number of trophoblast giant cells, but the continued generation of giant cells, presumably from trophoblast stem cells, is defective. To determine if the parthenote trophoblast cells albeit less abundant than in zygotic embryos, were functionally normal, we examined the expression of several genes important for normal trophoblast function. We first examined the trophectoderm of blastocysts for the cell adhesion molecule E-cadherin (uvomorulin). At the 8-cell stage, blastomeres increase adhesiveness and establish tight intercellular connections via E-cadherin. At the blastocyst stage, E-cadherin is expressed in all cells of the early embryo. Antibodies against E-cadherin disrupt these intercellular connections, leading to abnormal blastocyst formation (Shirayoshi *et al.*, 1983). Using immunocytochemistry, we detected E-cadherin in the both ICM and trophectoderm cells of parthenote blastocysts (Fig. 2a). We also examined the expression of E-cadherin in blastocyst outgrowths and found faint staining in giant cells and ICM cells of both zygotic and parthenogenetic outgrowths (data not shown). Thus, E-cadherin expression in zygotic and parthenogenetic blastocysts and blastocyst outgrowths was indistinguishable.
We next examined the expression in parthenotes of genes specific to the trophoblast developmental pathway. Hxt is trophoblast giant cell-specific basic helix-loop-helix transcription factor that is expressed in trophectoderm at the blastocyst stage, and in both proliferative and post-mitotic trophoblast giant cells. Hxt induces the differentiation of trophoblast stem cells into trophoblast giant cells in vitro, and of uncommitted blastomeres of cleavage stage mouse embryos to trophectoderm in blastocysts (Cross *et al.*, 1995). Like other basic helix-loop-helix transcription factors, such as MyoD (Weintraub, 1993), Hxt may determine the developmental program of the trophoblast (Cross *et al.*, 1995).

Whole-mount in situ hybridization demonstrated that Hxt expression in parthenote outgrowths was indistinguishable from that in zygotic blastocyst outgrowths (Fib. 2b). Placental lactogen-1 (mPL-1) gene is regulated by Hxt and produces a hormone secreted by trophoblast giant cells (Cross *et al.*, 1995). We saw intracellular mPL-1 in almost all giant trophoblast cells, but not ICM cells, in a peri-nuclear pattern by immunocytochemistry. However, no difference was found in the expression patterns between zygotic and parthenogenetic blastocyst outgrowths (Fig. 2c).

Proteinases are an essential part of the invasion program of trophoblast. Metalloproteinases, and their inhibitor, TIMP-1, are expressed by and are functional in peri-implantation embryos (Behrendtsen *et al.*, 1992; Brenner *et al.*, 1989). Because parthenote trophoblast is small and does not implant well into the maternal decidua, we next determined if the expression of gelatinase B, which is involved in invasion of the trophoblast, was expressed normally in parthenotes. Because zygotic outgrowth cultures have more trophoblast giant cells than do parthenote outgrowth cultures at day 5, but not at day 3, we used day 3 blastocyst outgrowths for these studies. The zygotic and parthenote blastocyst outgrowths secreted similar amounts of gelatinase B as determined by zymography (Fig. 3). There was no observable difference in the amount of activated gelatinase B between the two groups . Therefore, the parthenote trophoblast cells were normal for gelatinase B expression.

Parthenote and zygotic trophoblast giant cells differ in nuclear structure

Although the initial differentitaion of trophoblast giant cells was normal in parthenote, differences appeared with further time in culture. On careful observation, we noted that nuclear structure of parthenote trophoblast giant cells differed from that of zygotic trophoblast. We then used a polyclonal antibody that contained an autoantibody that reacted with nucleoli making them very obvious (Fig. 4a). We noted that trophoblast giant cells have either one or two or more nucleoli per nucleus. While zygotic and parthenote

giant cells had similar numbers of giant cells with a single nucleolus, zygotic outgrowths had significantly more trophoblast giant cells with more two or more nucleoli than parthenote outgrowths (Fig. 4b).

Parthenote giant cells show less DNA replication and have decreased viability in culture

Trophoblast giant cells do not undergo normal cell proliferation, but rather the genome replicates without cell division, a process called endoreduplication. Because the increased number and size of nucleoli may be an indication of less endoreduplication in the parthenote trophoblast giant cells, we examined the rate of DNA replication by measuring the uptake of the thymidine analog BUDR at day 3 of blastocyst outgrowth cultures, at a time when the number of giant cells is similar in the normal and parthenote cultures. After 1 or 2 hours of BUDR treatment, there was no significant difference between zygotic and parthenogenetic embryos in the percentage of total giant cells that were labeled with BUDR (Fig. 5). However, after 4 hours of BUDR treatment, more than 25% of the zygotic giant cells had labeled with BUDR, while only 12% of the parthenote giant cells had been labeled with BUDR. This was also true after 8 hours of BUDR treatment. Both giant cell types, those with one and those with two or more nucleoli incorporated BUDR. Thus, although there is the same number of giant cells in both parthenote and zygote cultures at this time, the parthenote giant cells incorporate less BUDR, suggesting that fewer of them are undergoing endoreduplication.

The continued life span of the parthenogenetic trophoblast giant cells in culture was also defective. We found that the parthenote outgrowth cultures did not survive as long in culture as the zygotic outgrowth cultures. The terminal outgrowths were characterized by the presence of vacuoles in trophoblast giant cells, thin trophoblast giant cells that appeared stretched to the point of breaking, and floating cells. We found that parthenote giant cells

became full of vacuoles by day 6. Soon after (day 6.5-7), the cells had become rounded and lifted off the plate, with only traces of giant cells left. When we counted the number of healthy parthenote and zygotic outgrowths and compared them to the total number of outgrowths, we found that significantly fewer of the parthenote outgrowths were viable than compared the zygotic cultures (day 6; 13/41parthenote outgrowths were viable, 14/15 zygotic outgrowths were viable). Although the day on which the parthenote cultures deteriorated was somewhat variable (from day 6 to day 8), in 5 experiments, we saw parthenote trophoblast giant cell always showed decreased viability in culture compared to zygotic trophoblast giant cells.

DISCUSSION

Parthenotes fail in early mouse development mainly because pjoor trophoblast differentiation or proliferation. We have shown that parthenote trophoblast giant cells behave normally in terms of initial differentiation in blastocyst outgrowth culture and initiation of expression of genes necessary for trophoblast function. However, the trophoblast giant cells of the parthenote embryos are deficient in proliferation, viability, nuclear morphology and endoreduplicative DNA replication.

Parthenote giant cells express appropriate trophoblast markers

Trophoblast differentiation is characterized by a program initiated by trophoblast-specific transcription factors such as the basic helix-loop-helix factor, Hxt, followed by the upregulation of trophoblast specific genes, such as gelatinase B and mPL-1, as well as continued expression of the more general ectodermal markers such as E-cadherin (Cross *et al.*, 1995; Cross *et al.*, 1994). Transcription of some trophoblast-specific genes, for example, mPL-1, is regulated by Hxt (Cross *et al.*, 1995). In our study, we have found

that trophoblast differentiation is initiated normally as shown by the expression of E-cadherin and Hxt, which are important for normal blastocyst and trophectoderm formation. The invasion program was also initiated normally as indicated by the production and activation of gelatinase B. Although fewer giant cells are produced by the parthenotes, initially the ones that are formed appear to express trophoblast-specific genes normally.

Parthenote trophoblast stem cells may be missing a proliferation signal

The parthenote embryos may have fewer giant cells because they are not replenished from trophoblast stem cells. Initially, parthenote and zygotic blastocyst outgrowths had the same number of trophoblast giant cells. However, after 5 days, the zygotic blastocyst outgrowths had significantly more trophoblast giant cells, while the parthenotes had the same number as at day 3. The increase seen after day 3 in zygotic cultures presumably was due to trophoblast stem cells differentiating into trophoblast giant cells, because the giant cells themselves do not proliferate, but instead undergo endoreduplication. We hypothesize that, as is the case for the inner cell mass (ICM) cells, the trophoblast stem cells are not maintained in parthenotes. ICM cells are the stem cells of the embryo and are not normally maintained in parthenotes, but can be maintained by agents that increase the proliferation of the stem cells, such as the Igf -1 receptor/ Igf-2 pathway, or leukaemia inhibitory factor (LIF) (Newman-Smith and Werb, 1995). The ICM produces a trophoblast proliferation signal that maintains proliferation of polar trophectoderm, but not mural trophectoderm. This pathway may function at the outgrowth stage as well, to increase proliferation of trophoblast cells near the ICM. In parthenotes, there is a cell-autonomous defect in trophoblast for responding to this signal (Gardner *et al.*, 1973). Unlike the Igf-1r/Igf-2 pathway in the ICM, this defect is not rescued effectively by materanl factors in vivo (Kaufman *et al.*, 1977).

The continued survival of the trophoblast giant cells, which was greatly reduced in the parthenotes, may also depend on the presence of a survival factor. Survival factors are

necessary for many cell types, such as neurons, to prevent the cells from undergoing programmed cell death (reviewed by (Raff, 1992). Parthenotes may be missing a survival factor present in zygotic trophoblast giant cells, and thus die. In other systems continued differentiated function also requires proper cell cycle gene regulation. The failure in DNA replication in parthenotes suggest that these parameters may be misregulated. Embryonic analysis of this sort suggests that there are growth signals important for trophoblast stem cell proliferation, as well as for trophoblast giant cell survival.

Trophoblast giant cells are defective in parthenotes

In addition to the lack of generation of trophoblast giant cells from precursors after 3 days in culture, the parthenote trophoblast giant cells were also defective in terminal differentiation as characterized by DNA endoreduplication. During differentiation of zygotic trophoblast, the trophoblast cells cease to divide, forming terminally differentiated, polyploid cells with DNA contents of up to 500N (reviewed in Hoffman and Wodding, 1993). However, when we examined parthenote giant cells we found that fewer parthenote giant cells incorporated BUDR, suggesting that fewer parthenote giant cells were undergoing endoreduplication. Because the parthenotes lack a proliferation signal for the ICM cells (Newman-Smith and Werb, 1995) and for the trophoblast stem cells (Barton *et al.*, 1985) , they may also lack a signal that induces the endoreduplicative cell cycle of the giant cells. Clearly a full understanding of the parthenogenetic trophoblast defect will require a detailed analysis of the endoreduplicative cell cycle, which, to date, has not been elucidated. Such studies are currently in progress.

The increased endoreduplication of the parthenogenetic trophoblast giant cells may be a characteristic of a later stage of differentiation of the giant cells, suggesting only two signals regulating trophoblast development, proliferation signal and a survival factor. We hypothesize that parthenotes lack secondary trophoblast giant cells, which arise from trophoblast stem cells instead of the trophectoderm of the blastocyst, in culture, and to

varying degrees in vivo, because the trophoblast stem cell population, like the ICM stem cell population is not maintained. Therefore, a lack of a trophoblast stem cell proliferation signal could lead to the later phenotype of less endoreduplication in trophoblast giant cells. Imprinted genes that increase proliferation, such as Mas, and the Igf-1r/Igf-2 pathway, or the unidentified signaling pathway from the ICM to the trophectoderm, may increase the proliferation of the trophoblast stem cells.

Therefore, a common element missing in the embryonic and extraembryonic lineages of parthenotes is regulation of DNA replication. Proliferation of stem cells for both lineages, and DNA endoreduplication of trophoblast giant cells appear to be defective in the absence of a paternal genome. Presumably, the lack of proliferation in trophoblast stem cells and of endoreuplication in trophoblast giant cells could be caused by the mis-expression of growth factors, growth factor receptors or transcription factors, necessary for trophoblast survival. The identification of such factors and whether they are regulated by genomic imprinting, would greatly further our understanding of trophoblast development in vivo.

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FIGURE LEGENDS

Fig. 1. Trophoblast giant cells in outgrowths from parthenote and zygotic blastocysts. (A) Representative photomicrographs of zygotic (left) and parthenote (right) blastocyst outgrowths after 5 days in culture. Bar indicates 50 μm. (B) Trophoblast giant cells in zygotic (solid bars) and parthenote (striped bars) blastocyst outgrowths after 3 or 5 days in culture. Day 3, zygotic 16 ± 4.3, n=19; parthenote, 17 ± 3, n=16; **p=**. Day 5, zygotic, 34.5 ± 2.5, n=24; parthenote, 13.0 ± 3.0, n=19; $p<0.001$. Values are expressed as mean ± s.e.m; Student's t-test was used to determine p values.

Fig. 2. Expression of E-cadherin, Hxt, and placental lactogen-1 in parthenote and zygotic blastocyst and blastocyst outgrowths. A. Immunocytochemistry with anti-E-cadherin antiserum was performed on zygotic blastocysts (left side) and parthenote blastocysts (right side) . Top row, phase contrast photomicrographs. Bottom row, fluroescence photomicrographs. B. Hxt expression was detected by in situ hybridization in zygotic (left side) and parthenogenetic (right side) outgrowths. Phase contrast (P/C) and brightfield (BF) photomicrographs are shown. C. Immunocytochemistry using mPL-1 antiserum was performed on zygotic (left) and parthenogenetic (right) outgrowths. Top row shows photomicrographs using phase-contrast microscopy while and the bottom row shows photomicrographs using fluorescent microscopy. Filled arrows point to giant cells expressing the Hxt or mPL-1 (as appropriate) while open arrows point to ICM cells that are not expressing Hxt or mPL-1. Bars indicate 50 μm.

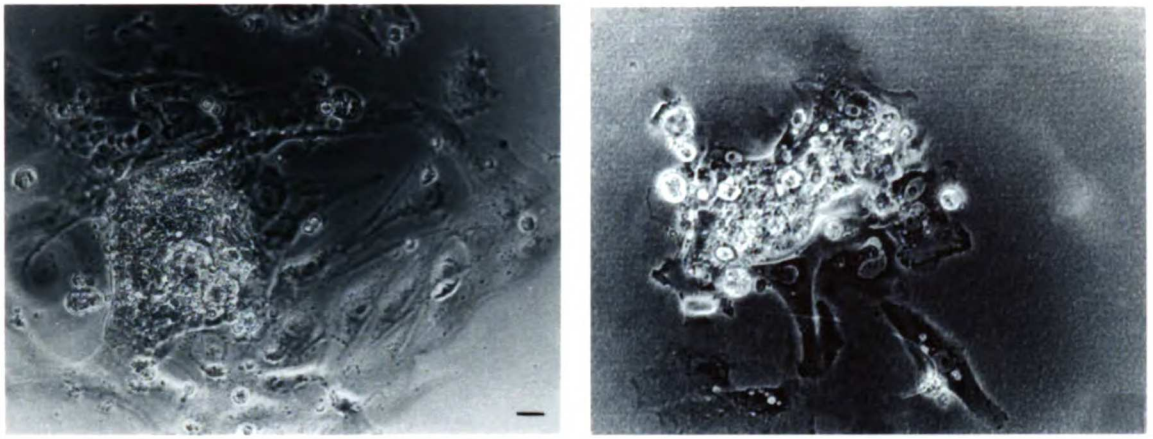
Fig. 3. Expression of gelatinase B in outgrowths from parthenote and zygotic blastocyst outgrowths. For these analyses, for each point, we placed 20 blastocysts in one large (20 μl) drop and analyzed the conditioned medium by zymography. Because fewer parthenote blastocysts attach and grow out (data not shown), we normalized gel loading to 5

outgrowths present at day 3. Conditioned medium collected from day 4 zygotic (Z) and parthenote (P) blastocyst outgrowths was collected and zymography performed. Closed arrow at left indicate the band corresponding to the proenzyme form of gelatinase B. Open arrow points to activated gelatinase B. Molecular weight standards ($x10^{-3}$) are indicated on the right.

Fig. 4. Nucleolar structures in parthenogenetic and zygotic trophoblast giant cells. A. Representative photomicrographs of day 3 zygotic (left) and parthenote (right) blastocyst outgrowths stained with an antibody that stains nucleoli. Filled arrow points to giant cells with one nucleoli per nucleus. Open arrow points to giant cells with two or more nucleoli per nucleus. Bar indicates 50 μm. B. The number of giant cells with 1 nucleolus were counted and compared to the number of giant cells with 2 or more nucleoli per nucleus. Zygotic and parthenote outgrowths had similar numbers of giant cells with one nucleolus. (Zygotic 24.9 ± 2.9 , n=24; parthenote, 20.2 ± 3.7, n=21.) Parthenote outgrowths had significantly fewer giant cells with two or more nucleoli than zygotic outgrowths. (Zygotic, 10.44 ± 1.3, n=25; parthenote, 5.2 ± 1.0, n=21; $p<0.005$.)

Fig. 5. DNA synthesis in parthenogenetic and zygotic trophoblast giant cells. Day 3 blastocyst outgrowth cultures were treated with BUDR for the indicated times. Cells that incorporated BUDR were labeled with an anti-BUDR antibody and visualized with fluorescence. After 1 or 2 hours of labeling with BUDR, zygotic (squares) and parthenote (diamonds) had similar numbers of giant cells positive for BUDR. (zygotic: 1 hr; 4.1 ± 1.4, n=8; 2 hr; 7.3 ± 2.4, n=11. Parthenote: 1 hr; 7.9 ± 2.8, n=9; 2 hr; 10.2 ± 2.9, n=12.) After 4 and 8 hr of BUDR labeling, fewer parthenote giant cells were positive for BUDR. (Zygotic: 4 hr; 27.2 ± 2.3, n=16; 8 hr; 24.3 ± 2.6, n=6. Parthenote: 4 hr; 13.0 ± 2.2, n=14, $p< 0.001$ compared to zygotic; 8 hr; 10.3 ± 3.0, n=5, $p= 0.006$ compared to zygotic.)

A



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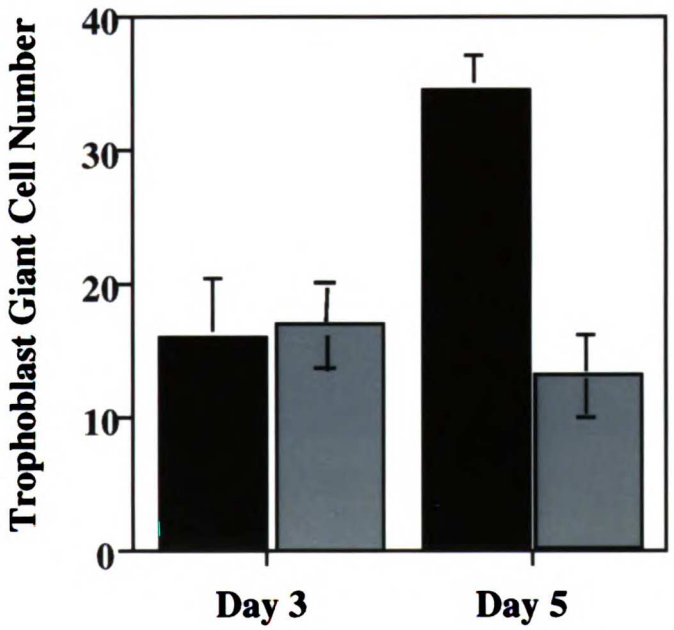


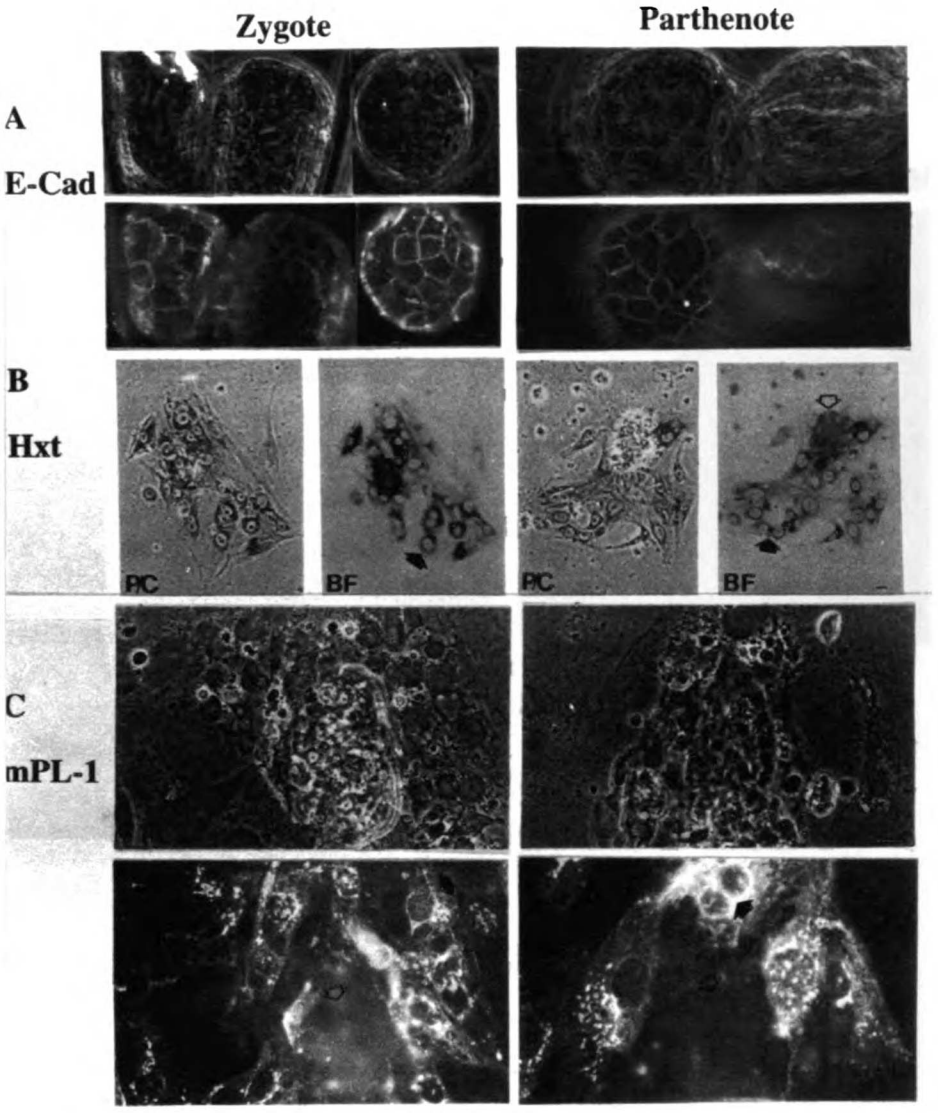


Figure 2

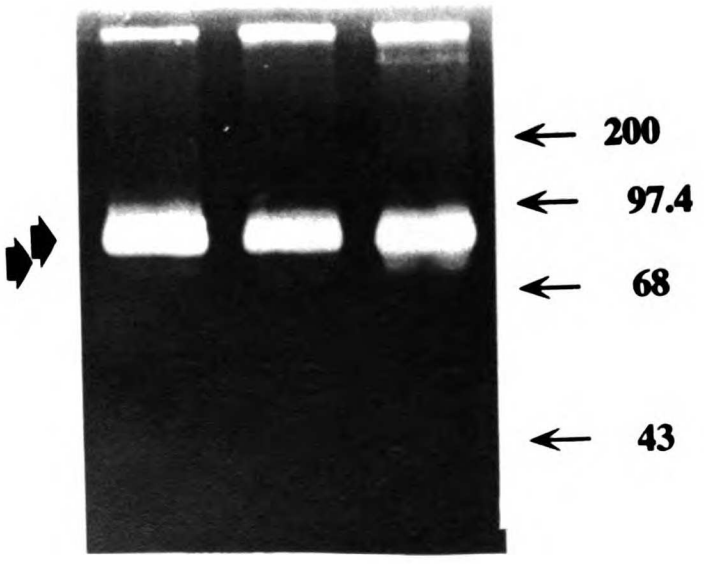
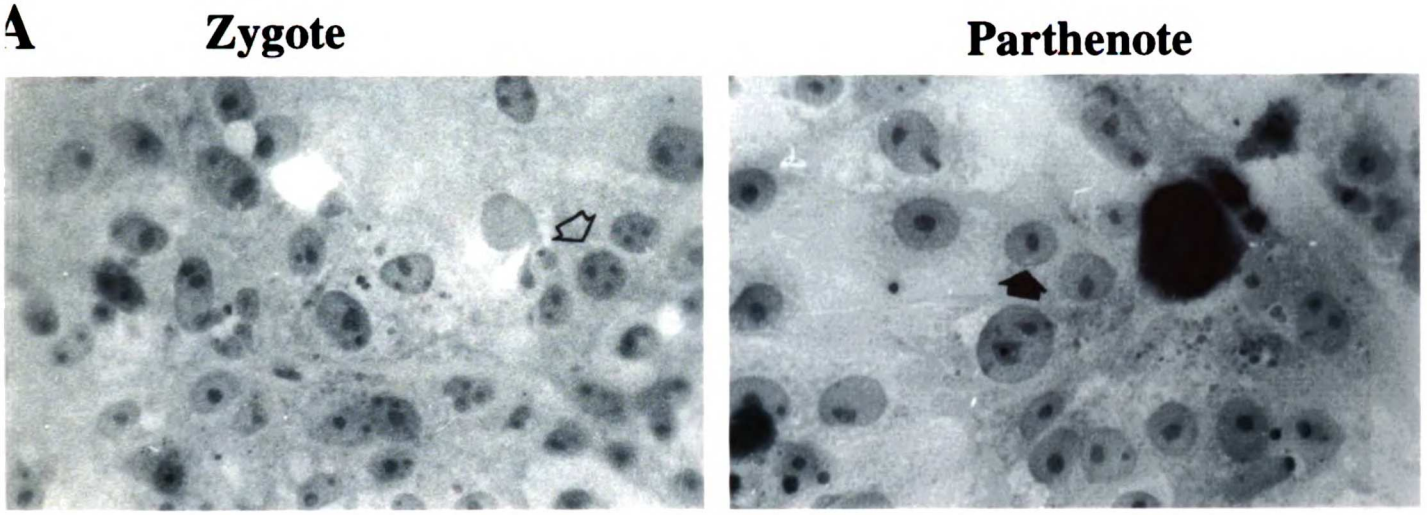
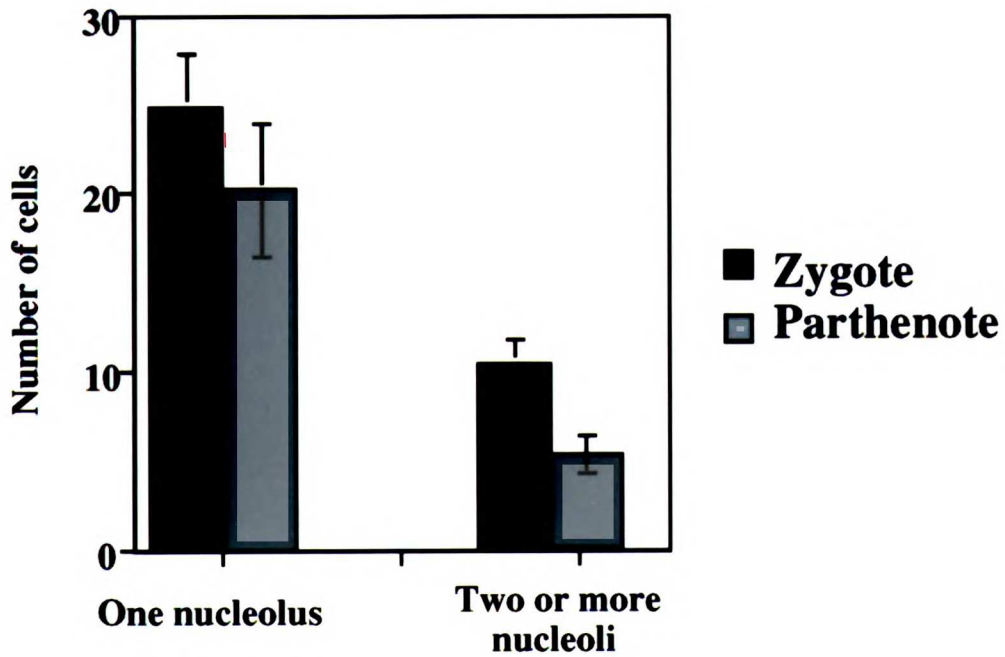


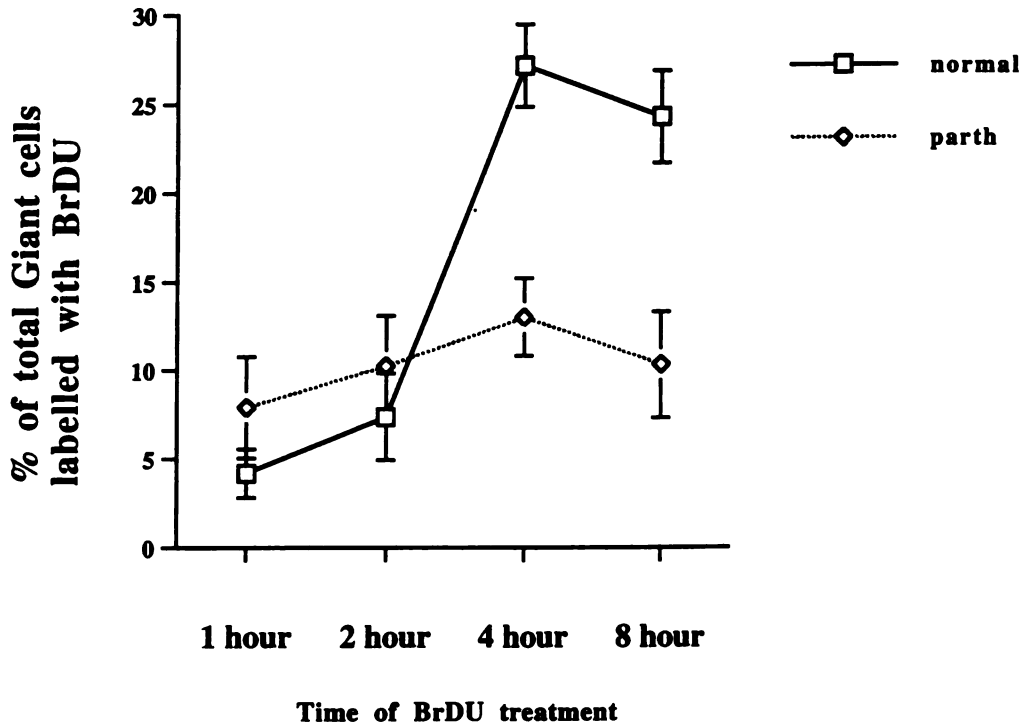
Figure 3

A


Zygote
Parthenote

B


30
20
10
0
Number of cells
One nucleolus
Two or more nucleoli
Zygote
Parthenote


% of total Giant cells labelled with BrDU
30
25
20
15
10
5
0
normal
parth
1 hour
2 hour
4 hour
8 hour
Time of BrDU treatment

Chapter 4:

Igf-1r expression in zygotic and parthenogenetic embryos

INTRODUCTION

Genomic imprinting regulates a variety of genes that function in early development (Bartolomei, 1994). Expression studies of imprinted genes have shown that they are not always expressed from only one allele, but that at certain times in development, or in certain tissues, imprinted genes are expressed from both alleles. For instance, Mas, a proto-oncogene with homology to the angiotensin receptor, is expressed exclusively from the paternal allele in all embryonic tissues tested up to embryonic day 12. By day 13.5, however, Mas is expressed from both alleles, except in heart, tongue and visceral yolk sac (Villar and Pedersen, 1994a, Villar and Pedersen, 1994b). Insulin-like growth factor (Igf) -2 is imprinted in all tissues, except in a small region of the brain, the leptomeninges (DeChiara, et al., 1991). These studies demonstrate that imprinting is tissue specific and developmentally specific.

Analysis of isoparental embryos implicates imprinted genes in key roles in early development. Embryos with only paternal DNA (androgenotes) or only maternal DNA (biparental gynogenotes or uniparental parthenotes) do not develop normally (Surani, et al., 1990). Androgenotes form small, disorganized embryos with large amounts of trophoblast, while parthenotes and gynogenotes display a range of embryonic phenotypes from almost normal, to sometimes just a ball of parietal endoderm, all with little trophoblast (Kaufman, et al., 1977, Sturm, et al., 1994). Presumably, the overexpression and underexpression of imprinted genes caused by having DNA from one parent is responsible for the developmental defects. However, which genes are responsible for these aspects of the developmental program is unknown.

I have been studying how imprinted genes regulate pre and peri-implantation development by characterizing the developmental defects in parthenotes, and testing whether genes not expressed in parthenotes are capable of rescuing these defects. In previous work, we showed that Igf-2 signaling through the Igf-1 receptor (Igf-1r) can

maintain the stem cell population of parthenote pre and peri-implanatation embryos (Newman-Smith and Werb, 1995). The Igf-1r (and Igf-2) are necessary in later development, from day 9 onward, to increase the growth in terms of size and weight of the developing embryo (Baker, et al., 1993, Liu, et al., 1993).

Although Igf-1r plays a role in cellular proliferation during early development, the regulation of its expression is unclear. I have shown using in situ hybridization and RT-PCR, that the Igf-1r is not expressed in parthenote blastocysts (Newman-Smith and Werb, 1995, Rappolee, et al., 1992). Although this suggests that the Igf-1r gene is imprinted at the blastocyst stage, a mouse strain made null for the Igf-1r by homologous recombination. shown no heterozygote phenotype, even when the deletion is inherited from the father (Liu, et al., 1993). If the Igf-1r gene were imprinted, and expressed only paternally, a heterozygote with the null allele inherited from the father would give the same phenotype as is seen in the homozygote. Because this is not the case for the Igf-1r, it is unlikely that the Igf-1r is imprinted, at least after embryonic day 9. In this chapter, I describe experiments that attempt to solve this disparity by determining if the Igf-1r is imprinted at the blastocyst stage.

The Igf-1r regulates cellular proliferation at two stages of development, the peri-implantation stage, and during the last half of gestation (Baker, et al., 1993, Newman-Smith and Werb, 1995). Because it is not imprinted at least from day 9 onwards, I hypothesize that it may be expressed in parthenotes after the blastocyst stage. It may be expressed in all peri and post-implantation parthenotes, or in only the ones that are phenotypically appear fairly normal at day 9. I tested this hypothesis by examining peri-implantation and post-implantation embryos for Igf-1r mRNA, comparing fairly normal to abnormal embryos.

MATERIALS AND METHODS

C57BL/6JxCBA/J)F1 mice (B6CBA) were obtained from Jackson Laboratories (Bar Harbor, ME). Pregnant mares' serum gonadotropin was purchased from Sigma Chemical co. (St. Louis, MO). Human chorionic gonadotropin (hCG) was obtained from Serono (Randolph, MA). Dulbecco's modified Eagle's medium (DMA) was prepared by the Cell Culture Facility (University of California, San Francisco). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT).

Embryo culture 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 mineral oil (Sigma) at 37°C with 5% CO^2 in air. Parthenotes were activated essentially as described by (Kaufman, 1978). Briefly, 1-cell embryos were isolated from superovulated B6CBA females and activated with 6% ethanol. The oocytes were then cultured for 5 hours in 1 µg/ml cytochalasin D to prevent extrusion of the second polar body. The embryos were washed extensively in FM-1 (Spindle, 1990) and cultured as described for the normal embryos. After the embryos had reached the expanded blastocyst stage, 3.5-4.5 days in culture, they were cultured in groups of 20-40 in 4-well Nunc culture dishes (for RNA isolation) or on tissue culture-treated plastic slides with 8 wells (LabTek) individually in drops (for in situ hybridization) in DME plus 10% FCS.

In situ hybridization

Whole-mount in situ hybridization was performed exactly as described previously (Newman-Smith and Werb, 1995).

RNA isolation

Pre-implantation embryos and blastocyst outgrowths were washed in phosphate buffered saline (PBS) and were lysed in GITC (Sambrook, et al., 1989). Samples were then frozen at -70°C and thawed at 37°C twice, and polyA mRNA was isolated using the Magic Bead kit (Promega). Briefly, samples were incubated with biotinylated oligo dT, and added to washed streptavidin-bound magnetic beads. The beads were collected in a magnetic stand, washed extensively. PolyA mRNA was eluted from the beads at 65°C for 2 minutes in water.

Post-implantation embryos were homogenized in GITC, and incubated with RNasol B (Biotex), and phenol/chloroform as described by the manufacturer. The aqueous layer was precipitated with EtOH, washed with 70% EtOH and resuspended in water.

RT-PCR

RT-PCR was performed basically as described (Rappolee, et al., 1988). RNA was reverse transcribed with Superscript II (BRL, Maryland) in 1X reaction buffer (supplied by manufacturer), 25 ng/ml oligo dT, 1.5 mM dNTPs at 45°C for 1 hour. The reaction was stopped by incubation at 65°C for 10 minutes. PCR was performed in a volume of 10-25 µl, using actin, GAPDH, or Igf-1r primers as described (Villar and Pedersen, 1994b). The PCR reaction mix contained 10 mM Tris-HCl (pH 8.3), 0.6 mM each dNTP, 10 µM of each primer, and variable amounts of Mg2+. For the Igf-1r we used an annealing temperature of 60°C with 2.5 mM Mg^{2+}, and for GAPDH and actin, we used an annealing

temperature of 65°C with 4.5 mM Mg^{2+}. For each set of primers, a reaction mix containing all components except enzyme was made, aliquoted and frozen. This reduced the amount of contamination.

RNAse protection assay

Mouse Igf-1r and GAPDH cDNAs were isolated using PCR, as described above. PCR fragments were cloned into pCRII using the TA Cloning kit (Invitrogen). The vectors were cut with the appropriate restriction enzymes to linearize, treated with phenol/chloroform, and ethanol precipitated. α–^{32}P-labeled transcripts were made by incubating the cut DNA with transcription buffer, NTPs, with UTP being labeled, and either SP6 or T7 polymerase at 37°C for one hour as described (Wilkinson, 1992). DNA was removed with RNAse free DNAse (BRL). The probes were purified on a 6% sequencing gel, and eluted out of the agarose overnight at 37°C (Sambrook, et al., 1989). The probe was added to the mRNA sample and precipitated with ethanol, and processes with the HybSpeed RPA kit or the RPA II kit according to the manufacturer's directions. For the HybSpeed RPA kit, hybridization buffer was added to the sample, and after 10 minutes at 68°C, treated with RNAse to digest unprotected fragments for 1 hour, and run on an analytical gel. For the RPA II kit, hybridization occurred overnight at 42°C, followed by RNAse treatment, and gel analysis. Gels were dried and exposed to X-ray film at -70°, or exposed to a Phosphor-imager cassette.

Analysis of Igf-1r imprinting

Embryos were isolated at different stages of development from Mus. spretus males mated with C57/B6 females by Dr. Angela Villar (Villar and Pedersen, 1994b). mRNA was isolated as described, and PCR performed essentially as detailed above. The cDNA was

digested with 10 units of the restriction enzyme Msp 1 at 37 °C for 3 hours to overnight, which cuts once within the *Mus domesiticus* (C57/B6) and twice within the *Mus spretus* Igf-1r cDNA PCR fragment (Villar and Pedersen, 1994b). The fragments were separated on 8% acrylamide gel in TBE buffer (Sambrook, et al., 1989). The gel was stained for 10 minutes in 5 μg/ml ethidium bromide, and DNA visualized and photographed under ultraviolet illumination.

RESULTS

I sought to determine if the Igf-1r was expressed at normal levels in peri- and post-implantation parthenogenetic embryos using three approaches: whole-mount in situ hybridization, RT-PCR and the RNAse protection assay.

Whole mount in situ hybridization:

Our previous work by whole mount in situ hybridization of parthenote blastocysts demonstrated that the Igf-1r was expressed at very low levels, if at all, in the parthenote blastocysts (Newman-Smith and Werb, 1995). To determine if the Igf-1r was expressed in the peri-implantation or post-implantation stages, blastocyst outgrowths were analyzed by whole mount in situ hybridization (Wilkinson, 1992). To control for non-specific signals, two approaches are commonly taken; using sense probes, or using antisense probes for different mRNAs. As shown in Fig. 1, both zygotic and parthenogenetic blastocyst outgrowths expressed similar, low levels of Igf-1r mRNA. Although the levels were low, the Igf-1r mRNA localization was different from the control signal, the insulin receptor, suggesting that the Igf-1r signal was specific. However, further studies need to be done with several different control probes to ensure that the low level of staining is specific to Igf-1r and is not background staining.

RT-PCR

Because the levels of Igf-1r mRNA were very low in normal and parthenote blastocyst outgrowths as determined by in situ hybridization, the sensitive method of RT-PCR was used to detect Igf-1r mRNA (Rappolee, et al., 1988). At the blastocyst stage, Igf-1r mRNA was detected in zygotic, but not parthenogenetic blastocysts, after 30 amplification cycles when the sensitivity of the RT-PCR reaction was increased by Southern blotting (Fig. 2). After 60 amplification cycles Igf-r was detectable in the parthenogenetic blastocysts, although the signal was always weaker than that seen in zygotic blastocysts. These results demonstrate that the Igf-1r is expressed in parthenotes, but at a much lower level than in zygotic embryos.

Previous results have shown the ES/LIF medium is able to correct one of the defects of parthenotes by maintaining the stem cell population of the ICM (Newman-Smith and Werb, 1995). Because the Igf-1r is not imprinted at day 9 (earliest time point analyzed), it is possible that the Igf-1r gene expression comes on in parthenotes at later stages. Therefore, mRNA collected from later stage embryos was analyzed by RT-PCR. Because parthenogenetic blastocysts grown in ES/LIF medium develop large ICMs, comparable to zygotic embryos, and perhaps develop to later stages than when grown in DME medium, we examined Igf-1r expression in parthenogenetic blastocyst outgrowths grown both in DME medium (with 10% FCS) and in ES/LIF medium. In preliminary experiments using RT-PCR, Igf-1r mRNA was detected in zygotic blastocysts and day 3 zygotic blastocyst outgrowths (growth in ES/LIF), and in parthenogenetic blastocyst outgrowths, but not in parthenote blastocysts (Fig. 3a). The results are suggestive because the relative levels of Igf-1r were as expected; low in the zygotic blastocysts, not present in the parthenogenetic blastocysts after 30 cycles (as seen in other experiments, such as Fig 2.), and much higher levels in the outgrowths, which contain significantly more cells than the blastocysts. These results were observed for several different experiments. To control for possible mRNA degradation during sample preparation and

reverse transcription, duplicate samples were analyzed for Actin mRNA. All samples contained actin (Fig. 3b). The zygotic and parthenogenetic blastocysts had amplified bands of similar intensity, as did the zygotic and parthenogenetic blastocyst outgrowths. This suggests that the relative levels seen in the Igf-1r PCR reaction may reflect different levels of Igf-1r mRNA. It also suggests that the parthenogenetic blastocyst sample does not contain a general PCR inhibitor, which would account for the lack of detection of Igf-1r mRNA. Igf-1r was also detected in a sample that contained no mRNA, indicating contamination, a common problem in PCR. However, amplification of Igf-1r from a sample with no mRNA is not acceptable. This contamination was eradicated by obtaining new, unopened reagents, and making aliquots of the PCR mix in a different laboratory (Fig. 3c).

I also analyzed later stage parthenogenetic embryos that developed in vivo. RT-PCR was performed on day 9 parthenotes that developed fairly normally, and of several parthenotes that developed poorly as shown in Fig. 4a. Day 7.5 parthenogenetic and zygotic embryos were also analyzed. The results of a typical experiment are shown in Fig. 4b. Igf-1r was detected in both types of day 9 parthenogenetic embryos, ones that developed well and ones that developed poorly. However, in this experiment (but not in others) Igf-1r was not detected in normal embryos. In addition, Igf-1r was detected even if no reverse transcriptase was added to the RNA during the RT reaction, suggesting contamination. However, when no RNA was added to the RT reaction, no Igf-1r was detectable. Likewise, when no RT sample was added to the PCR reaction, no Igf-1r was detected. Therefore, the RT and PCR reagents were not contaminated, suggesting that the mRNA samples themselves were contaminated. It is interesting to note that in this case, unlike that described above, Igf-1r was detected in samples that did not have RT enzyme in the RT reaction, but not in samples that did have RT enzyme. Therefore, the RT enzyme, or a byproduct of the enzyme activity (such as using up the nucleotides?) inhibited the PCR

reaction. Therefore, although these results suggest that parthenote blastocyst outgrowths express the Igf-1r gene, they are not definitive.

RNAse Protection Assay

The RNAse protection assay was used to determine if the Igf-1r was expressed in post-implantation embryos. This has several characteristics that make it more suited to these specific experiments. The samples already collected for use in RT-PCR can be used for this assay. This is especially important for the in vivo collected parthenogenetic embryos which were difficult to obtain. Even though these samples were shown to be contaminated with DNA, the RNAse protection assay does not amplify the Igf-1r signal, and a small amount of DNA will not be visible. The RNAse assay hybridization step occurs in liquid phase, and thus the amount of signal is directly correlated to the amount of mRNA (Sambrook, et al., 1989). By using the Phosphor-Imager, rather than X-ray film (the density of the band is corresponds to the intensity of the signal only over a short range of intensity/density) and a densitomiter, a wide range of mRNA concentrations can be directly compared. Quantitative PCR is possible, but much more difficult, and not as convincing. The greatest problem with the RNAse protection assay is that it is not as sensitive as PCR, and thus, more sample must be used.

The human Igf-1r probe that had been used for in situ-hybridization of blastocysts and outgrowths was initially used for the RNAse protection assay. However, it proved to be too different from the mouse sequence, and did not work (data not shown). I used PCR to clone the mouse Igf-1r (from both spretus and domesticus species) into pCRII, which has suitable promoters for making mRNA probes. Only the domesticus probe was suitable with domesticus mRNA (data not shown). Two different RNAse kits (plus a non-kit protocol) are available. The RPA II kit is similar to the basic protocol, and included an overnight hybridization. Hybridization using the second kit, the hybespeed kit, was only 10 minutes. As well as decreasing the time of the assay, this also decreases the time

mRNA is kept at a high temperature, where RNAses may degrade it. The hybridization buffer in the hybespeed kit is under patent, and its contents are not disclosed, although it does not contain hydrophobic exclusion agents such as dextran. The two different kits were directly compared (Fig. 5). The hybespeed kit gave a much stronger signal for GAPDH than did the RPA II kit. As mentioned above, human Igf-1r probe does not detect mouse Igf-1r, although, as expected, it did protect a PCR fragment amplified from human DNA. However, when both GAPDH and Igf-1r probe were added to the same tube, two bands are easily detected. One of these bands is slightly higher than the GAPDH protected fragment, and one of these is slightly higher than the Igf-1r protected fragment. This suggests that the two probes are interacting with each other, perhaps through the portions of the polylinkers that are the shared in the two vectors. This necessitated using these probes separately.

In order to determine how sensitive the RNAse protection assay was, a range of concentrations of mRNA from both adult liver and day 15 embryos were tested (Fig 6). One ug of embryonic, but not adult, was sufficient to detect Igf-1r.

The early embryo samples were then tested for Igf-1r mRNA using the RNAse protection assay. I used GAPDH to normalize the samples, so that direct comparisons could be made, based on the ratio of Igf-2r mRNA to GAPDH mRNA. The results of one such assay are shown in Fig. 7. GAPDH mRNA was detectable in every sample. The Igf-1r was not detectable in all samples, although a strong signal is seen in zygotic day 15.5 embryos. However, protected probe is detectable in samples containing parthenogenetic day 9.5 embryonic mRNA. These results suggest that the Igf-1r is expressed in parthenotes that develop beyond the blastocyst stage. Unfortunately, the zygotic sample for day 9.5 was degraded, so no direct comparison between zygotic and parthenogenetic samples was possible.

Imprinting status of the Igf-1r

To determine the imprinting status of the Igf-1r in blastocysts, I used the system worked out by Angela Villar, a graduate student in Roger Pedersen's lab (Villar and Pedersen, 1994a, Villar and Pedersen, 1994b). She used interspecies crosses and identified restriction enzyme differences, which she used to identify the two alleles. By performing RT-PCR on isolated F1 hybrid mRNA samples and then cutting with restriction enzymes, she was able to determine is expression was from one allele or both. In previous work, she analyzed the Igf-1r gene for imprinting in the day 1 neonates and found that the Igf-1r was not imprinted at this stage (Villar and Pedersen, 1994b). Using mRNA from day 12.5 F1 hybrid embryonic yolk sac, I performed PCR and restriction analysis and determined that the Igf-1r was not imprinted at this stage in this tissue, i.e. it was expressed bi-allelically (Fig. 8). I obtained F1 hybrid blastocysts (5 blastocysts total), as well as day 6.5 and day 7.5 embryonic and ectoplacental cone samples from Dr. Angela Villar and Dr. Roger Pedersen, and isolated polyA mRNA. As shown in Fig. 4b, these samples contained DNA, as determined by the band when no RT was added, and when RT was added, PCR was inhibited, similar to the PCR experiments described above. Thus, the imprinting status of the Igf-1r in pre and peri-implantation embryos is still unknown.

DISCUSSION:

The expression of the Igf-1r in parthenotes is paradoxical. We previously detected none, or very low amounts, in parthenote blastocysts (Newman-Smith and Werb, 1995). However, other experiments show that it is not imprinted from midgestation to birth (Baker, et al., 1993, Liu, et al., 1993). I analyzed the expression of Igf-1r mRNA in pre, peri- and post-implantation embryos. I tested the hypothesis that the Igf-1r was expressed

in parthenotes at a later stage of development, and compared the expression in parthenotes that developed fairly normally to those that developed very abnormally.

The expression of Igf-1r is very low in peri- and early post-implantation embryos

Using whole-mount in situ hybridization, I was able to detect Igf-1r mRNA in zygote derived blastocysts. Using the same technique, I was able to detect very low amounts of Igf-1r mRNA in zygotic and parthenogenetic day 3 peri-implantation embryos, significantly lower than other mRNAs, such as Rex-1 (Chapter 2), Hxt (Chapter 3), and the insulin receptor (data not shown). Although further experiments need to be done to confirm that the signal is specific, these results suggest that the Igf-1r is expressed at very low levels in early implantation zygotic and parthenogenetic embryos.

I also examined Igf-1r mRNA expression using the RNAse protection assay. I examined blastocysts, day 7.5, day 9.5 and day 15 embryos. Igf-1r was clearly present in day 15 zygotic embryos, and faint bands were present in day 7.5 and day 9.5 zygotic embryos. A faint band was also present in day 9.5 parthenogenetic embryos. GAPDH mRNA was readily detectable in these samples. This again suggests that Igf-1r is expressed at low levels in early post implantation embryos.

Although the level of Igf-1r mRNA is very low, we were able to detect the expression in parthenotes at two stages, in the blastocyst, and at day 9.5. Some Igf-1r mRNA was detected in parthenote blastocysts using RT-PCR, but only after 60 cycles of amplification. For zygotic blastocysts, we were able to detect a signal after 40 cycles, and the signal at 60 cycles was stronger for the zygotes than for the parthenotes. Therefore, while the parthenotes express the Igf-1r, they do so at a lower level than in zygotic embryos.

At day 9.5, parthenogenetic embryos again expressed Igf-1r mRNA. However, we are unable to conclude that the parthenotes are now expressing the Igf-1r at normal levels without testing a range of zygotic and parthenogenetic embryonic mRNA concentrations. The parthenote sample had eight times as much mRNA, so its possible that the amount of zygotic mRNA was too low, and that the parthenotes are expressing Igf-1r mRNA at normal levels. From these studies, I can conclude that parthenotes express the Igf-1r at day 9.5 at low levels, but I can't conclude if the Igf-1r gene is expressed at normal levels in parthenotes.

Is the Igf-1r imprinted at the blastocyst stage?

Unfortunately, we still do not have an answer to this question. PCR contamination was an constant problem. I managed to solve the problem of contamination of RT and PCR components by making mixes containing all the necessary components (except enzyme) in a different lab using new components. This lead to the conclusion that the RNA samples were becoming contaminated with DNA during preparation. This is not surprising since I was working on PCR while making RNAse protection probes and in situ hybridization probes for Igf-1r as well as constructs for injecting into embryos. Therefore, Igf-1r RNA and DNA were abundant in the lab. In order to repeat these experiments, it would be necessary to isolate the mRNA again. I attempted to do this by mating spretus males to CBA females, and obtaining blastocysts. However, even when I superovulated the CBA females, I had few successful matings and was not able to obtain any blastocysts. I also tried using natural matings, again with no success.

However, it is possible that the Igf-1r is not expressed in parthenote blastocysts for reasons other than imprinting. It is possible that a regulator of Igf-1r expression is imprinted. This would not be detected in the homologous deletion of the receptor because the regulator would be expressed in the heterozygote, causing expression from the allele

that is present. It is also possible that one of the developmental defects of parthenotes leads to mis-expression of the Igf-1r. Perhaps a developmental program (such as the one leading to normal trophoblast development) is not initiated, and this prevents Igf-1r from being expressed.

Further analysis using the type of experiments described here is necessary to conclude if the Igf-1r is imprinted at the blastocyst stage, and its expression levels relative to zygotic embryos. however, these experiments do demonstrate that parthenogenetic embryos express Igf-1r mRNA during peri- and post-implantation development.

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FIGURE LEGENDS

Figure 1. Igf-1r mRNA expression in blastocyst outgrowths. Igf-1r mRNA was detected by whole-mount in situ hybridization. Left column, zygotic outgrowths. Right column, parthenogenetic outgrowths. P/C, photomicrographs using phase contrast microscopy. BF, photomicrographs using bright-field microscopy.

Figure 2. Igf-1r mRNA expression in blastocysts. RT-PCR, followed by southern blotting was performed on 5 normally fertilized (N) or parthenogenetic (P) blastocysts, or liver mRNA (+) using primers specific for Igf-1r for 30 or 50 cycles. Autoradiograph shows detection of bands by human Igf-1r probe. Arrow marks correct location of the Igf-1r amplified product. Lower bands are primers. The lane marked 0 had no RT mix in the PCR reaction.

Figure 3. Igf-1r and actin mRNA expression in blastocysts and day 3 blastocysts outgrowths. RT-PCR was performed on 5 blastocyst (B) and 2 blastocyst outgrowths (O, bottom row in A) using Igf-1r (A, C) or actin (B) primers for 50 cycles. For the actin RT-PCR, samples were run in duplicate. Correct location of amplified products shown by arrows. N, normally fertilized; P, parthenogenetic. C. Diagnostic RT-PCR. +: liver mRNA in RT mix; - RT: no RT enzyme added. -RNA, +RT: no RNA added to RT mix containing RT enzyme; 0: no RT added to PCR mix; MW: molecular weight markers.

Figure 4. Igf-1r expression in vivo. A. Photomicrographs of day 9.5 in vivo parthenotes. Left side; Fairly normal parthenote with visible axis. Right side; severely abnormal parthenote, basically only a ball of extraembryonic cells. B. 50 cycles of RT-PCR was performed on embryos that developed in vivo. Lanes 1-5, reverse transcriptase was present in the RT reaction. Lanes 6-12, reverse transcriptase was not present in the RT reaction. Lane 1, 6, day 7.5 parthenote. Lane 2, 7, severely abnormal day 9.5

parthenote. Lane 3, 8, fairly normal day 9.5 parthenote. Lane 4, 9, day 7.5 zygotic embryo. Lane 5, 10, day 9 zygotic embryo. Lane 11, no RNA in RT mix. Lane 12, spretus/musculus day 7.5 F1 hybrid. Lane 13, no RT mix in PCR reaction.

Figure 5. Comparison of Hybespeed and RPA II Rnase Protection Assay kits. Lanes 1-5 were processed using the Hybespeed kit. Lanes 6-9 were processed using the RPA II kit. Lanes 1, 6: undigested Igf-1r probe. Lanes 2, 7: undigested GAPDH probe. Lanes 3, 8: 10 μg liver mRNA with GAPDH probe. Lanes 4, 9: 10 μg liver mRNA with Igf-1r and GAPDH probes. Lane 5: Igf-1r PCR product probed with Igf-1r as positive control.

Figure 6. Igf-1r detection in adult and embryonic tissue. 1 or 10 μg of day 15 pc embryonic mRNA was hybridized with GAPDH or Igf-1r as indicated. 0.5, 1 or 5 μg adult liver mRNA was hybridized with GAPDH (G) or Igf-1r as indicated.

Figure 7. Detection of Igf-1r mRNA in embryos. Normally fertilized (N) and parthenogenetic (P) embryos at the blastocyst stage (blast, B), 7.5, 9.5, or 15.5 (+) days p.c. were analyzed by RNAse protection assay for Igf-1r or GAPDH, as indicated. Arrows mark site of relevant protected fragment.

Figure 8. Imprinting status of the Igf-1r at day 12.5 in visceral yolk sac. mRNA from *Mus domesticus* (dom) and F1 *Mus spretus/ Mus domesticus* hybrid day 12.5 visceral yolk sac (V. Ys) was analyzed by RT-PCR, and restriction digested with Msp1. Large arrows point to bands from domesticus mice, while small arrows point to bands from *Mus spretus* embryos.

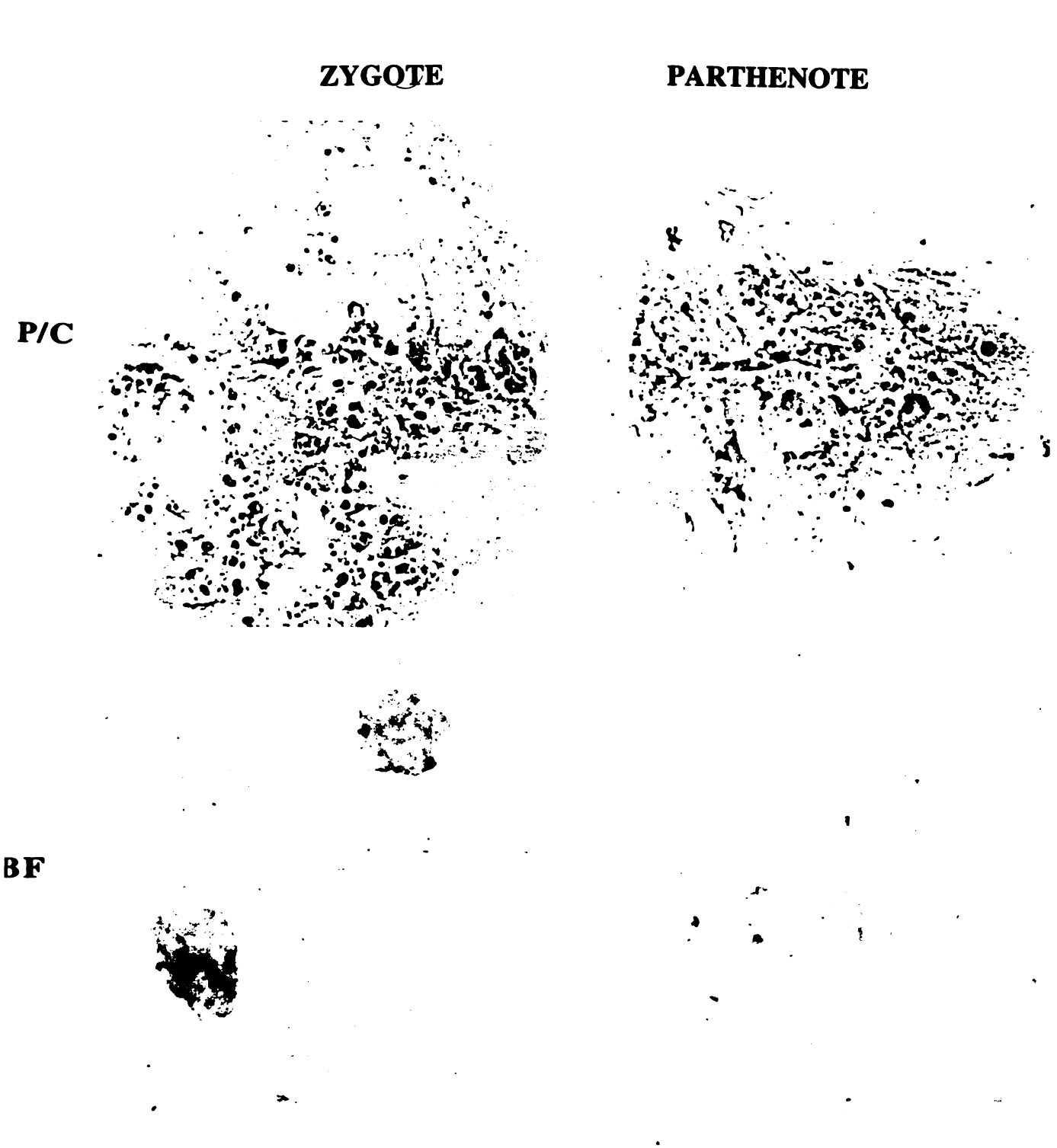

Fig. 1

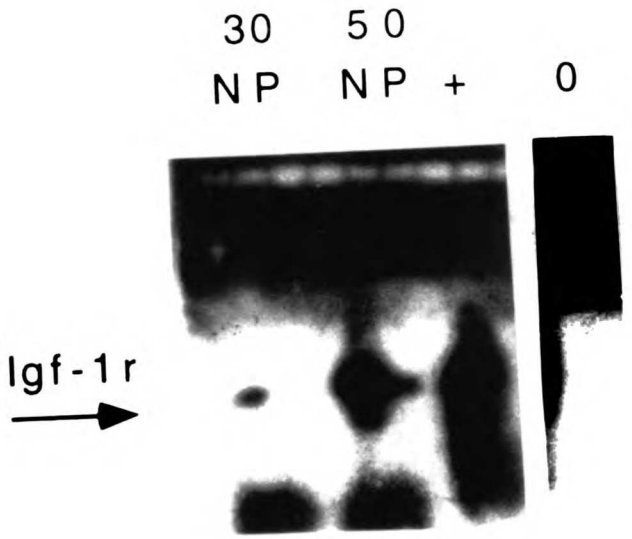

Figure 2

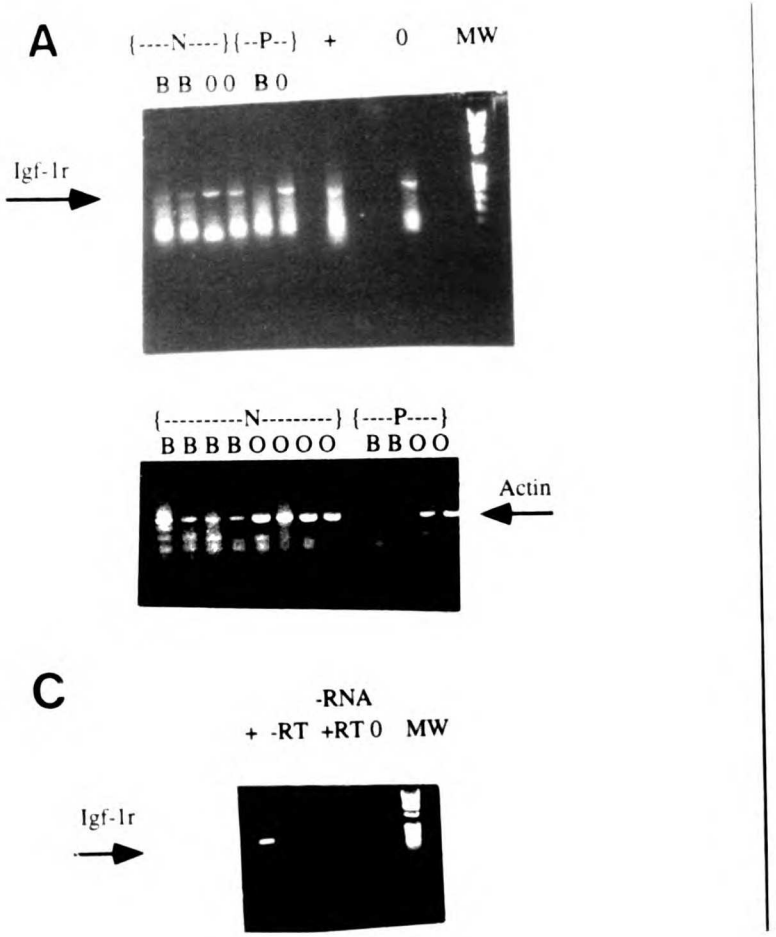

Figure 3

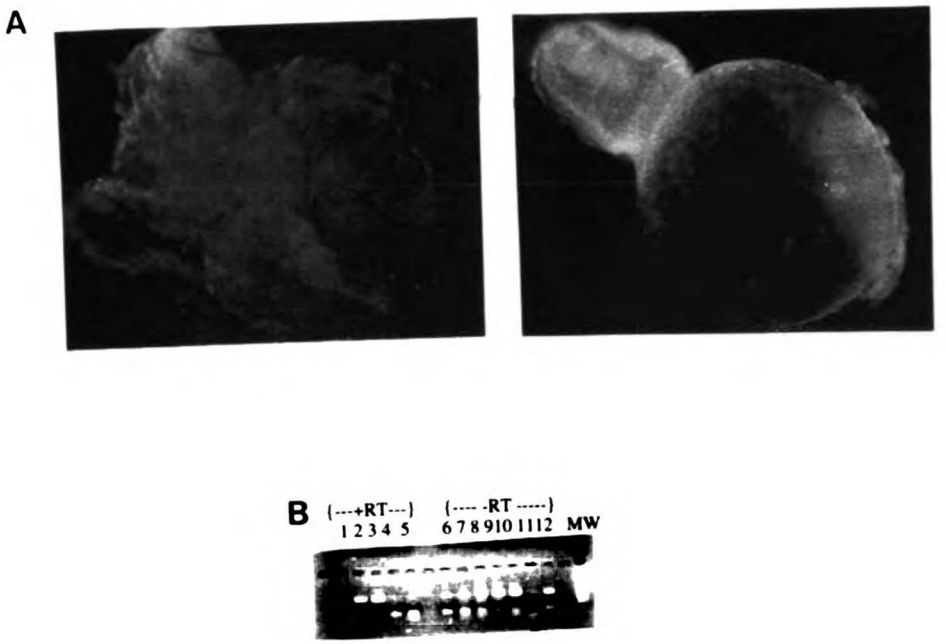

Figure 4

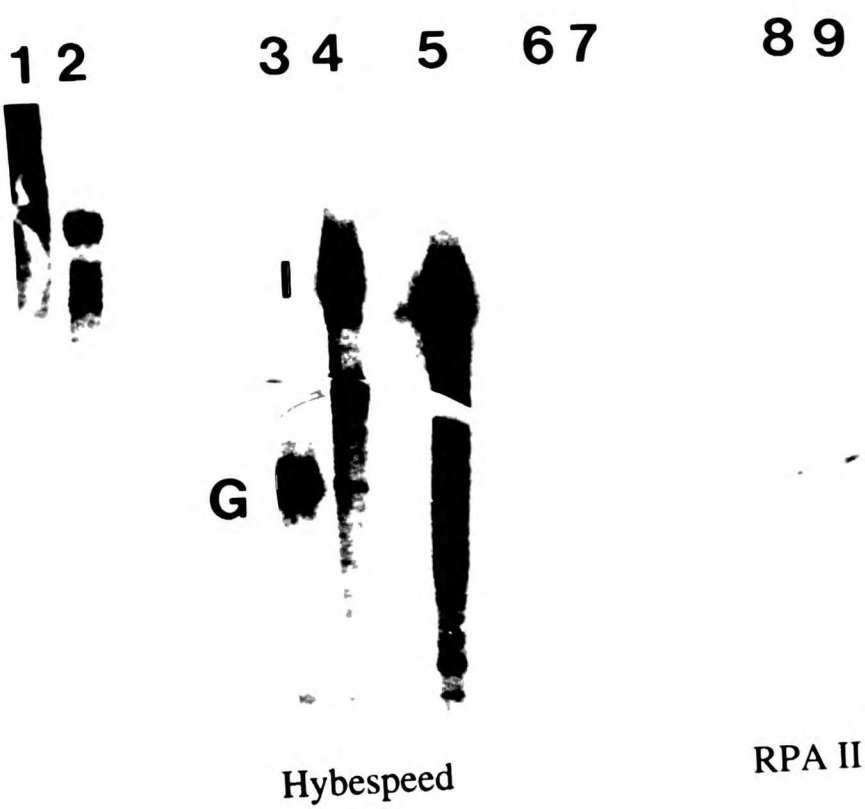

Figure 5

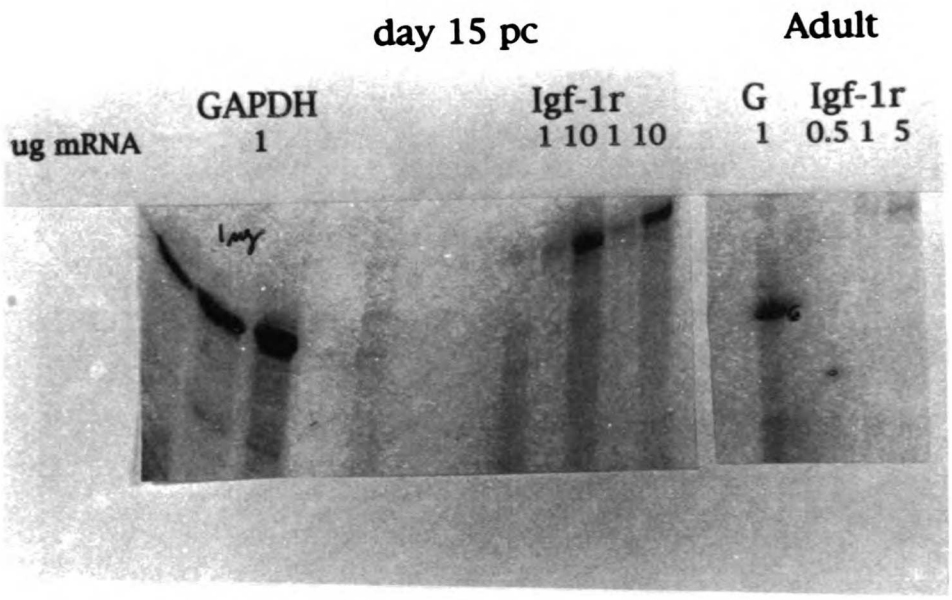

Figure 6

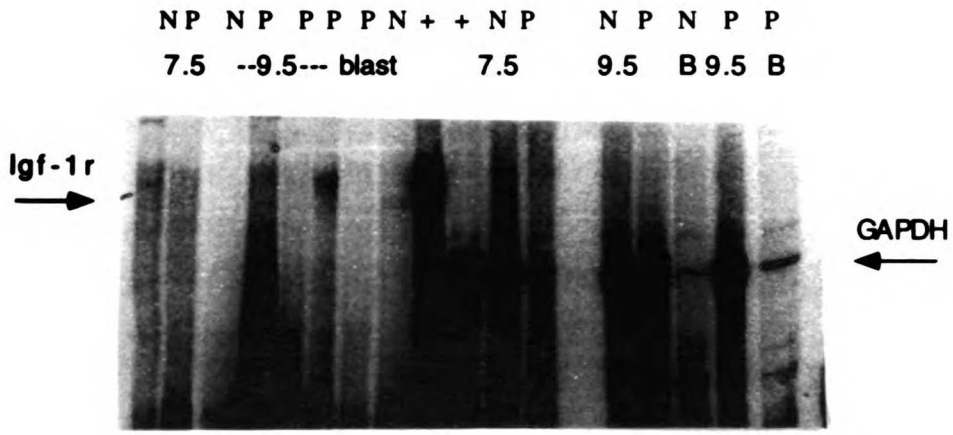

Figure 7

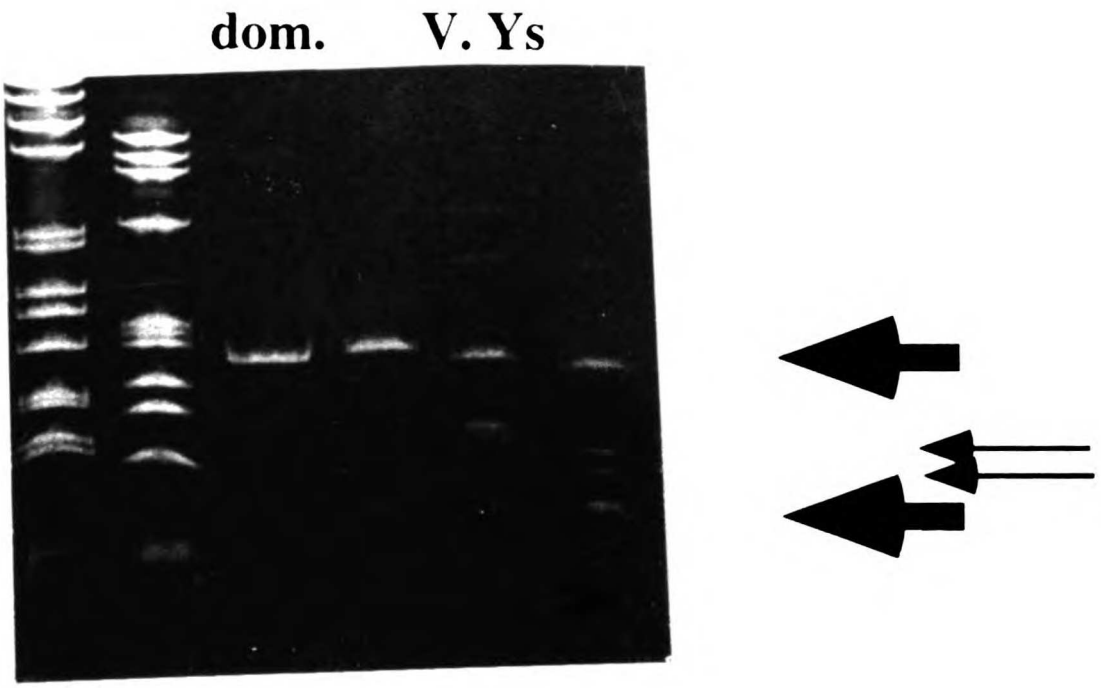

Figure 8

Chapter 5:

Signals controlling peri-implantation development in the mouse

INTRODUCTION

By examining parthenogenetic embryos, we have demonstrated the presence of several signals that are necessary form normal development, and are misregulated in the parthenotes. The results from this work, and others, suggests a model of pre- and peri-implantation development. At the blastocyst stage, the embryo produces two proliferative signals, one secreted by the inner cell mass (ICM) that acts on trophectoderm, and one that acts on the ICM. These signals continue to function throughout peri-implantation development. At this stage, the ICM can differentiate into visceral endoderm (VE) or parietal endoderm (PE). Different factors affect the stem cell decision of which differentiation pathway to follow. Development of the trophoblast is also controlled at this stage by two, or perhaps three, different signaling pathways. As in the blastocyst stage, a proliferation signal from the ICM induces proliferation of the trophoblast stem cells. A survival factor keeps the trophoblast giant cells alive. The third signal may act like a proliferation factor, directly increasing endoreduplication, or it may induce differentiation of trophoblast stem cells into trophoblast giant cells that endoreduplicate at a higher rate.

ICM DEVELOPMENT

At the blastocyst stage, the embryo grows in response to two signals (Fig. 1). One signal is secreted from the ICM and acts on trophectoderm to increase proliferation (Gardner et al., 1973). Another signal increases the proliferation of ICM cells. The evidence for a proliferation signal at the blastocyst stage comes from examining parthenogenetic embryos. Parthenogenetic ICM stem cells do not proliferate normally, leading to a vastly decreased stem cell population. We have shown that parthenote blastocysts do not express insulin-like growth factor (Igf) -2 (Igf-2) or the Igf-1 receptor (Igf-1r) at normal levels. Because Igf-2 is a mitogen, and signals through the Igf-1r, activating the Igf-1r kinase (Furlanetto et al., 1987; Sara and Hall, 1990), we tested the

hypothesis that the ICM stem cells do not proliferate because they are missing the Igf-1r/Igf-2 signaling pathway by injecting one cell parthenotes with expression constructs for the Igf-1r, treating the blastocysts with Igf-2, and counting the number of ICM cells. We found that the Igf-2/Igf-1r did, in fact, increase the proliferation of, and therefore the number of, ICM cells in the blastocyst to the normal level. This, however, is not the only signaling pathway that leads to proliferation of the ICM because mice without Igf-2/Igf-1r can develop past this stage. It is not surprising that there is redundancy at this stage, because, without enough pluripotent stem cells, the embryo would not survive.

This proliferation signal for the ICM stem cells is also functional at the peri-implantation stage. Parthenote blastocyst outgrowths, or ICM outgrowths, do not maintain the stem cell population, while zygotic outgrowths do. We again tested whether Igf-2/Igf-1r could restore the proliferation of parthenogenetic ICM stem cells, and found that this signaling pathway could indeed restore proliferation. Because we knew from the Igf-2/Igf-1r null mice that this was not the only proliferation pathway, we tested whether LIF, which is produced by the mother at the site of implantation, could also induce proliferation in the ICM stem cells. This maternal factor was able to maintain the stem cell population, and may function as a proliferation factor in the early embryos of the Igf-1r/Igf-2 null mice, and with Igf-2/Igf-1r in normal embryos.

At the peri-implantation stage, the proliferating stem cells differentiate into PE or VE, depending on the signals that they receive. In parthenotes, the ICM cells differentiate predominately into PE cells, while zygotic embryos differentiate into PE, VE, and at later stages, other cell types. The predominance of PE cells in parthenotes suggests three scenarios. The first scenario invokes a developmental clock, with stem cells differentiating first into PE, then, after a certain amount of time, or a certain number of cell divisions, differentiating into VE. In this case, the parthenotes would run out of stem cells before VE was formed. This scenario predicts that if the parthenotes had sufficient stem cells, VE would be formed. We tested this by treating blastocyst outgrowths with LIF, leading to the

formation of large ICMs, and then removing the LIF to allow the outgrowths to differentiate. The parthenote ICMs still differentiated predominantly into PE, even though ICM stem cells were maintained throughout the experiment. These results suggest that PE and VE differentiation are not controlled by a developmental clock.

If PE and VE differentiation are not controlled by a developmental clock, the differentiation pathways may be controlled by two different signals (Fig. 2). In one scenario, the parthenotes are pushed down the PE pathway, perhaps by synthesizing too much of a differentiation factor, or too much receptor, too little of an antagonist/inhibitor. In another scenario, the parthenotes are blocked from the VE differentiation pathway. In the former scenario, more PE cells than normal would be made, because the PE pathway is enhanced. We found however, that the parthenote outgrowths had the same number of PE cells as zygotic outgrowths. This suggests that a VE inductive signal is missing, so that the parthenotes do not make VE.

TROPHOBLAST DEVELOPMENT

Trophoblast development, like ICM development is controlled by proliferation signals. At the blastocyst stage, the ICM expresses a proliferation signal that induces proliferation of the polar trophectoderm, the trophectoderm cells that are nearest to the ICM, (Ilgren, 1983). Analysis of chimeric parthenogenetic/zygotic embryos demonstrated that this signaling pathway is disrupted in parthenotes. When parthenote ICMs were transferred into zygotic trophectoderm vesicles, the resulting embryo developed normally. However, when zygotic ICMs were transferred into parthenogenetic trophoblast vesicles, the resulting embryo developed as poorly as parthenogenetic embryos (Barton et al., 1985). Thus, parthenote ICMs secrete the proliferation signal for trophectoderm, but parthenote trophectoderm has a cell autonomous defect that prevents it from responding to this signal. This suggests that the trophectoderm is missing a receptor, or some other component of the signaling pathway. Two receptors are known to be missing in

parthenotes: Igf-1r and Mas, a protooncogene that shares homology with the angiotensin receptor [Villar, 1994 #164]. It is possible that one of these receptors is responsible for mediating the trophectoderm proliferation signal from the ICM.

At the peri-implantation stage, trophoblast development requires some type of cell survival signal, as well as a signal that leads to increased endoreduplication. Both of these pathways are impaired in parthenotes. Trophoblast giant cells of parthenotes die in culture earlier than their zygotic counterparts, suggesting some kind of pathway that is misregulated in parthenotes. The parthenotes may be missing a survival factor, or may overexpress a cell death factor. H-19, which is expressed in parthenotes presumably at twice the normal level, can act as a tumor suppressor, probably because H19 inhibits transcription of Igf-2 (Hao et al., 1993; Leighton et al., 1995). Mammalian cells transfected with expression constructs for H-19 grow slower than mock-transfected cells, but the cells do survive, suggesting that overexpression of H19 in parthenotes is not responsible for trophoblast giant cell death (Hao et al., 1993). Perhaps parthenotes are missing a survival factor. Signaling through the Igf-1r (by Igf-1) has been shown in some systems to be a survival factor (Raff, 1992). Igf-1r, activated by Igf-2, may have a similar effect in this system. However, the Igf-1r/Igf-2 null mice do not have a trophoblast phenotype in vivo, suggesting that either the Igf-1r/Igf-2 pathway is not involved, or that other factors present in vivo, but not in vitro, play this role (Baker et al., 1993; Liu et al., 1993). It is also possible that other imprinted genes, as yet undiscovered, are necessary for trophoblast giant cell survival.

Our model also hypothesizes a signal that increases endoreduplication in trophoblast giant cells, based on examination of parthenote development. More zygotic trophoblast giant cells are endoreduplicating than parthenogenetic trophoblast giant cells. The simplest model to explain these data is that the parthenotes lack an endoreduplication signal that is present in the zygotic embryos. This suggests that three signals control trophoblast development, a proliferation signal, a survival factor, and an endoreduplicative signal (Fig.

3). Alternatively, increased endoreduplication may be a characteristic of a later stage of differentiation of the giant cells, suggesting only two signals regulating trophoblast development, proliferation signal and a survival factor (Fig. 4). In fact, data examining nuclear structure of trophoblast giant cells, suggests that, while trophoblast giant cell differentiation is initiated normally in parthenotes, it is stalled after a certain point, leading to less differentiated trophoblast. This may be because the parthenotes lack a population of trophoblast giant cells, the secondary giant cells, that arise from trophoblast stem cells instead of the trophectoderm of the blastocyst. We hypothesize that parthenotes lack these in culture, and to varying degrees in vivo, because the trophoblast stem cell population, like the ICM stem cell population is not maintained. Therefore, a lack of a trophoblast stem cell proliferation signal could lead to the later phenotype of less endoreduplication in trophoblast giant cells. Imprinted genes that increase proliferation, such as Mas, and the Igf-1r/Igf-2 pathway, or the unidentified signaling pathway from the ICM to the trophectoderm, may increase the proliferation of the trophoblast stem cells.

The ICM and trophoblast of the early embryo both require proliferation signals to maintain their stem cell populations. If either of these is perturbed, the embryo will not grow. Other signals are important for the differentiation of specific cell types. Our model postulates five signals that direct the development of the early embryo (Table 1). Most of these were identified in vitro. Are they relevant in vivo?

DEVELOPMENT IN VIVO

Most often, in vivo as in vitro, the parthenotes develop into balls of PE which are secreting large amounts of laminin (see figure 5) (Sturm et al., 1994). However, some parthenotes develop somewhat normally to the early limb bud stage in vivo(Kaufman et al., 1977; Sturm et al., 1994). In vitro, I used a simplified culture system to determine at which stages parthenogenetic embryos failed. The case of a developing parthenote in vivo is

much more complicated. The blastocyst expresses receptors for a variety of growth factors, such as insulin, EGF, FGF, TGFa, and LIF and some of these growth factors are present in the maternal environment (Rappolee and Werb, 1993). Indeed, we have shown that LIF is able to compensate for the lack of Igf-2/Igf-1r in the parthenotes. Other factors may be able to compensate for other missing steps in parthenotes. The success of the trophoblast stem cell proliferation may be the biggest determinate of parthenogenetic success, because chimeric embryos between parthenotes and tetraploid embryos (which preferentially inhabit the trophoblast lineage) develop much more often into normal embryos at day 12 (A. Spindle, K. Sturm, M. Flannery and J. J. Meneses, R. A. Pedersen, personal communication). Perhaps some parthenotes develop well in vivo because their trophoblast stem cells proliferate more normally than other parthenotes. The blastocyst, and embryo, express so many growth factor receptors, many of which induce proliferation, suggesting that several compensatory pathways exist to ensure the early development of the parthenotes. In some embryos, trophoblast stem cells may be stimulated sufficiently by non-imprinted pathways to develop more normally. Why some parthenotes do well and some don't probably depends on cues from the maternal environment.

IMPRINTING FROM AN EVOLUTIONARY VIEWPOINT

Genomic imprinting is a major regulatory mechanism in development of viviparous mammals. How did this regulatory mechanism arise? What are the evolutionary forces that led to the establishment and to the maintenance of genetic imprinting? One obvious maintenance force is that parthenogenesis is prevented, and only sexual reproduction is successful. However, this is also true for species that do not regulate genes by imprinting, and so is not the only evolutionary pressure that maintains imprinting in mammals.

Imprinting may have originated as an extension of a host defense system against foreign DNA (Barlow, 1993). In mouse embryos, foreign DNA, such as retroviruses, is

methylated when inserted into the genome. This prevents the expression of key genes required for viral production, thus saving the host from infection. In the same way, imprinted genes are marked by methylation, which affects their transcription. Methylation fulfills the criteria for an imprinting mark because it is an epigenetic modification, is heritable, erasable, and modifies transcription (Bird, 1992). Imprinted genes, then, may contain sequences that are similar to foreign DNA, and thus are methylated. Alternatively, foreign DNA may have inserted near imprinted genes, or within imprinted genes (for example, in Igf-2 receptor), and the subsequent methylation of this DNA led to the differential transcription we describe as imprinting. In support of this model is the fact that some transgenes, which often contain bacterial DNA, become methylated (Surani et al., 1988). This methylation can have the effect of inhibiting transcription, as in the case of the TG.A transgene, or of stimulating transcription, as in the case of the Igf-2r (Chaillet et al., 1991; Stoger et al., 1993). Because methylation can inhibit or stimulate transcription, and because the oocyte contains large amounts of methyltransferase, while sperm does not, imprinting may act in the maternal line only (Li et al., 1992). Indeed, all transgenes are hypermethylated on the maternal allele, suggesting that methylation occurs in the maternal line.

This model suggests that genes became regulated by imprinting by chance, depending on the site of foreign DNA insertion, or if their sequence resembled foreignDNA. But what are the evolutionary forces that maintained some genes as imprinted, while (presumably) not others? The evolutionary drive to reproduce sexually is present in animals that do not imprint genes, suggesting that this is not the only evolutionary force that maintains imprinting. One hypothesis, that agrees with Barlow's theory that imprinting is a maternal mechanism has been dubbed the "Ovarian Time Bomb" hypothesis (Varmuza and Mann, 1994). This hypothesis stipulates that imprinting is a protection against malignant trophoblast disease that would otherwise occur when oocytes are spontaneously activated in the ovary. The mammalian embryo invades the maternal

tissues so extensively that trophoblast growth must be strictly controlled. The females are protected from the trophoblast of spontaneous parthenotes by inactivating genes that are necessary for trophoblast development. In human molar pregnancies, which arise from enucleated eggs that have been fertilized, malignant trophoblast disease occurs 1000 times more often than does a normal or ectopic pregnancy. Perhaps if oocytes did not repress trophoblast growth genes by imprinting, most ovarian tumors would become malignant trophoblast tumors, and kill the mothers.

This hypothesis predicts that imprinting is a maternal regulatory mechanism, but does not predict a paternal imprinting mechanism. At the time this theory was postulated, no paternal specific methylation patterns were discovered that were present in sperm, so as to be an imprinting signal. Now, however, H19 has been shown to display parental specific methylation patterns, with the paternal allele being more methylated, and some of these methylation sites are present in the sperm (Ferguson-Smith et al., 1993). This suggests that, if methylation is the imprinting signal, that during spermatogenesis, an imprinting signal is applied to the paternal allele. These results refute the notion that imprinting only occurs in the maternal gametes. However, it is not known if the methylation sites of H19 are indeed imprinting signals.

But what about the imprinted genes that are not involved in trophoblast growth, but for example are involved in embryonic (ICM) growth, or in unrelated processes, such as mRNA splicing? This theory's most controversial point is that these other genes are imprinted by accident, because they contain similar signals that are recognized by the imprinting machinery. Perhaps these genes became imprinted by a foreign gene inserting nearby, and are not being selected for evolutionarily, but simply have not yet lost their imprinting status. This seems unlikely. It is more probable that these other genes are imprinted for reasons other than repressing trophoblast growth. This does not necessarily refute the theory, but it does limit its scope.

Another hypothesis , put forth by Moore and Haig, also invokes trophoblast growth as an evolutionary force for maintaining imprinting (Moore and Haig., 1991). This hypothesis sees imprinting as mediating a prenatal tug of war via the evolutionarily fitness of the mother and the evolutionary fitness of the father. For the most of the mothers genes to be passed on, she should have large litters. The father on the other hand, will be more evolutionarily fit if his offspring outcompete the offspring of other males in the same womb now, or at a later pregnancy. Therefore the male will do best if his offspring are large, at the expense of other litermates, or at the expense of the mother, which will prevent later pregnancies with other males. One problem with this theory is that if the male makes its offspring too large, the mother may die either before, or during parturition. This would not be beneficial for the neonate, since it would lack food. Alternatively, if the mother were successful in decreasing the size of the embryos, they may be too small to survive. Perhaps the evolutionarily pressures that maintain imprinting are not an embryonic tug of war between the parental genomes, but rather a balancing act to obtain the most beneficial size of embryo.

Some imprinted genes fit the model of competition between embryonic genomes. Igf-2, when not present, leads to smaller animals, and when overexpressed, leads to larger animals. Igf-2 is expressed paternally, according to this theory, to increase the size of the father's pup, but not maternally, which results in smaller size of all pups, allowing for more pups per liter. The Igf-2r, binds Igf-2 and targets it for destruction, thus decreasing the amount of Igf-2. This gene is expressed maternally, to decrease Igf-2 further, but is not expressed paternally. Other imprinted genes discovered since this theory was published also affect embryonic growth. H19's primary function may be to inhibit the transcription of Igf-2. In H19 null mice, the only obvious phenotype is larger mice (Leighton et al., 1995). However, this phenotype is not observed if the H19 mice are crossed with Igf-2 null mice. Maternal expression of H19, then, leads to less Igf-2, and

smaller mice. The Ins2 gene is expressed paternally in the yolk sac, and may benefit the paternal offspring by increasing the size of the placenta (Giddings et al., 1994).

Some imprinted genes, however, do not take part in the tug of war between the parental geneomes. Mash2 is expressed maternally, and without this gene, trophoblast development is impaired (Guillemot et al., 1994). Thus, the mother expresses a gene necessary for trophoblast growth, not the father. Sp6 and Snrpn are involved in mRNA splicing, and Snrpn is expressed predominantly in the brain (Hayashizaki et al., 1994; Leff et al., 1992). How these could affect embryonic growth is unknown. Therefore, as in the last hypothesis, not all the imprinted genes can be explained.

Doubtless, as more imprinted genes are discovered, more hypotheses as to the evolutionary drive to maintain imprinting will be put forth. Perhaps as the mechanism of imprinting is discovered, and the catalogue of imprinted genes expanded, a unifying theory will emerge. At this point, its difficult to develop a theory that can account for the imprinting of splicing factors, growth factors, and genes that function as RNAs. I contend that imprinted genes have initially became imprinted by chance, perhaps as a consequence of foreign DNA inserting into the genome nearby, and through evolution, individual genes maintained that imprinting for individual reasons, some to prevent asexual reproduction, some to regulate embryonic size and some to prevent malignancies in the maternal ovary. Just as genes that are regulated by other mechanisms, such as post translational modification, or by transcrptional activation with SP6, have a variety of roles and functions in the animal, so to may imprinting regulate different genes for different evolutionary reasons. It is not necessary for all genes that are imprinted to be maintained in this state for the same evolutionary reason.

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Figure Legends:

Figure 1. Proliferation signals at the blastocyst stage. One signal (1) increases the proliferation of ICM stem cells. The Igf-2/Igf-1r signaling pathway can increase ICM cell proliferation in parthenotes, which do not express this signaling pathway at normal levels. The ICM secretes another proliferation signal (2) that increases proliferation of the polar trophectoderm, but not the mural trophectoderm.

Figure 2. Growth and differentiation signals of the ICM at the peri-implantation stage. The ICM stem cells are maintained by a proliferation signal that may be Igf-2/Igf-1r, LIF or other maternal factors. The ICM expresses two differentiation signals, one that induces VE cells (2), and one that induces PE cells. ICM cells grown on a substratum of LN also induces VE cells, while ICM cells grown on a substratum of FN, or treatment with PTHr-P induces PE cells.

Figure 3. One model of signals affecting trophoblast differentiation. The ICM secretes a proliferative signal at this stage, as at the preimplantation stage. A survival factor (2) acts upon the trophoblast giant cells to maintain viability. The embryo may also synthesize another factor (3) which directly increases endoreduplication of the trophoblast giant cells.

Figure 4. An alternative model of trophoblast differentiation. As in figure 3, a proliferative signal (1) from the ICM is hypothesized. This increases the proliferation of the diploid trophoblast cells, which then differentiate into 2° giant trophoblast cells, which are characterized by increased endoreduplication. A survival factor (2) increases the viability of the 1° trophoblast giant cells.

Figure 5. Laminin secretion by zygotic and parthenogenetic ICMs. Zygotic (A, C) and parthenogenetic (B, D) blastocysts were grown in LIF containing medium as described in chapter 2. The ICMs were isolated and allowed to differentiate for 2 days, and immunocytochemistry with an anti-LN antibody was performed. A and B are phase contrast photomicrographs, while C, and D are fluorescent photomicrographs. Arrow points to the ICM cells, which are stained strongly with the LN antibody in the parthenogenetic, but not the zygotic ICM.

Table 1. Factors that function in pre and periimplantation development. The role the signal plays in development is indicated, along with possibilities as to what the factors may be.

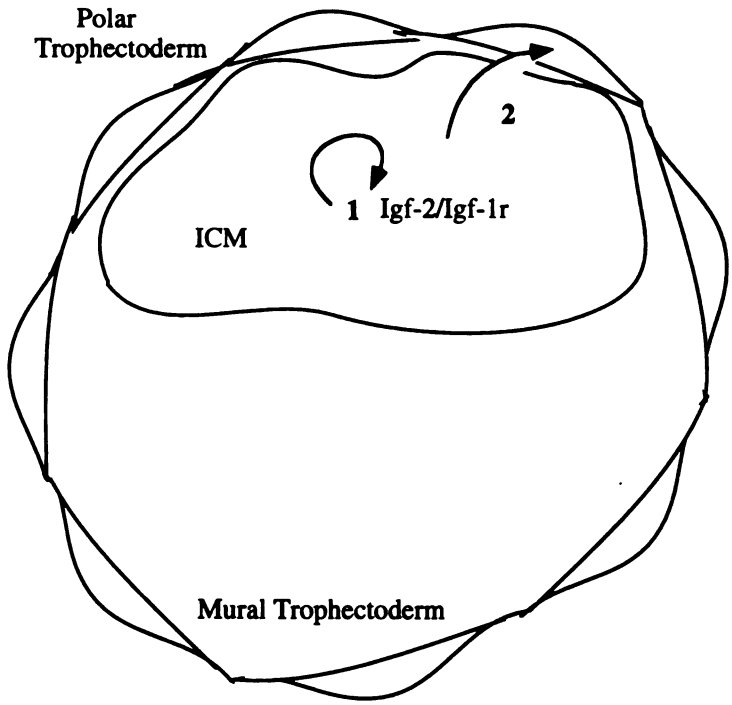
Polar
Trophectoderm
2
1 Igf-2/Igf-1r
ICM
Mural Trophectoderm

Fig. 1

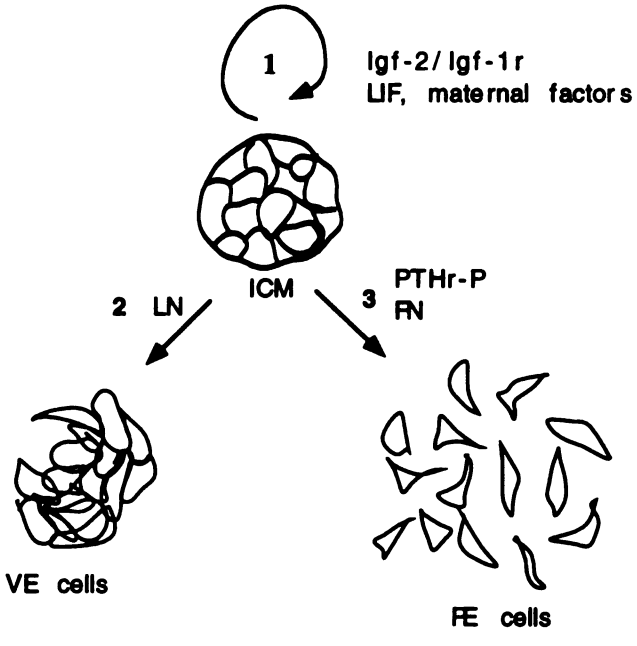
1
Igf-2/Igf-1r
LIF, maternal factors
ICM
2 LN
3 PTHr-P
FN
VE cells
PE cells

Fig. 2

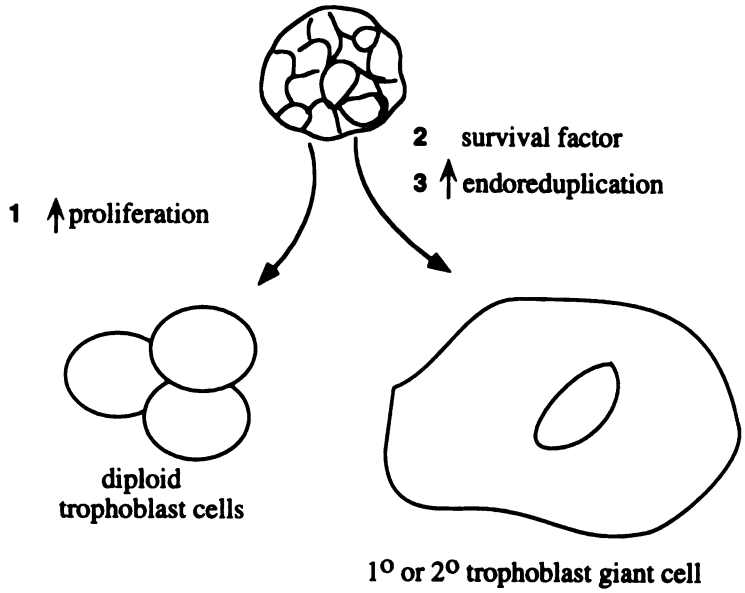
2 survival factor
3 ↑endoreduplication
1 ↑proliferation
diploid
trophoblast cells
1° or 2° trophoblast giant cell

Fig. 3

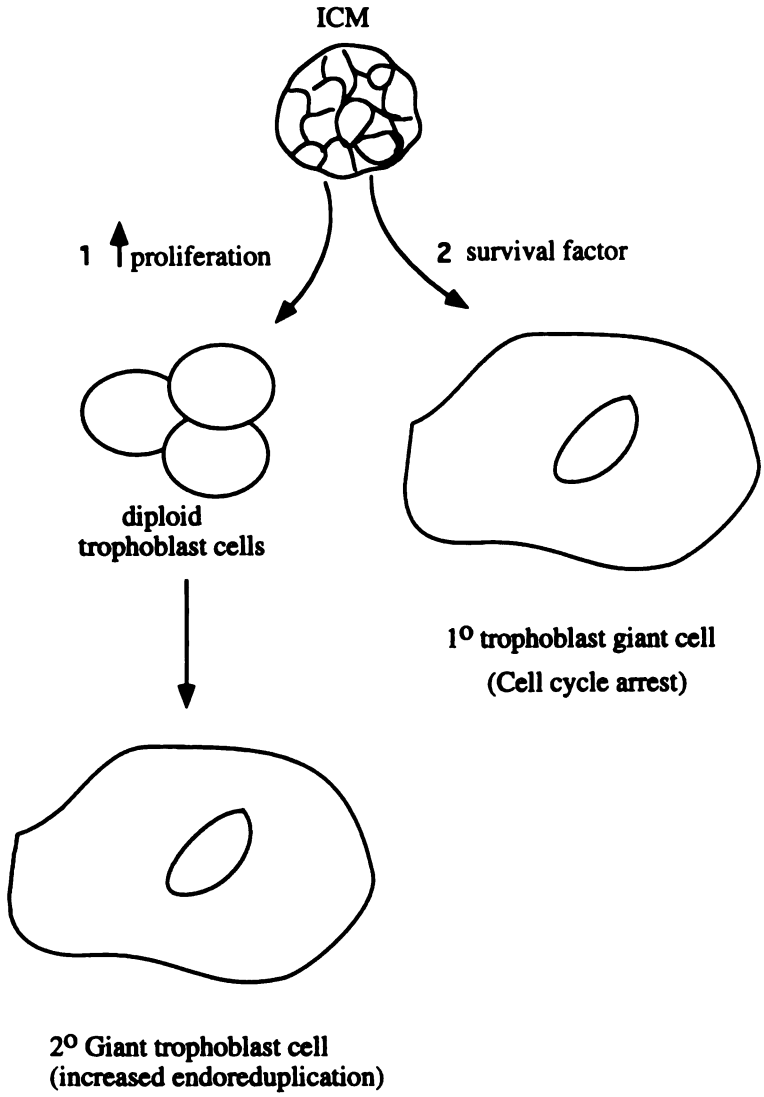
ICM
1 proliferation
2 survival factor
diploid
trophoblast cells
1° trophoblast giant cell
(Cell cycle arrest)
2° Giant trophoblast cell
(increased endoreduplication)

Fig. 4

Parth ICMs express LN

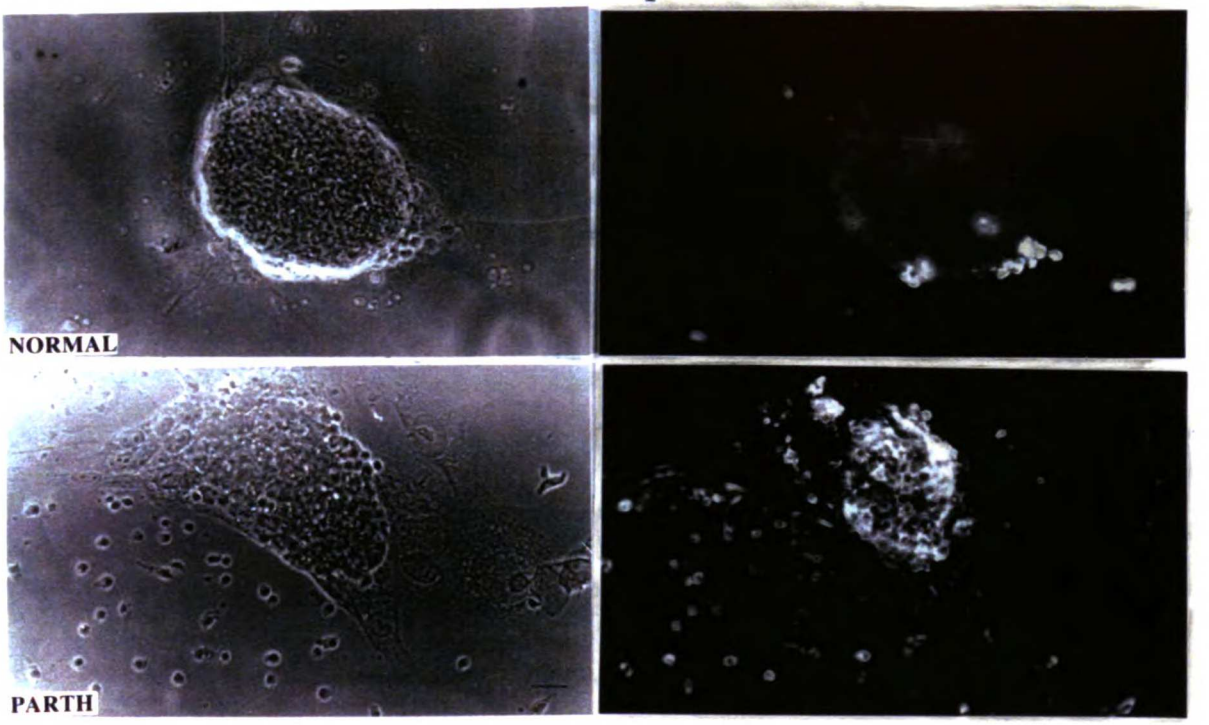

Figure 5

#	Role	Factor
1	ICM prolifertion	Igf-2/Igf-1r, LIF
2	VE differentiation	?
3	Trophoblast stem cell proliferation	signal from ICM
4	Trophoblast giant cell survival	?
5	Trophoblast giant cell endoreduplication	?