

Influence of chromosomal determinants on development of androgenetic and parthenogenetic cells

M. AZIM SURANI, SHEILA C. BARTON, SARAH K. HOWLETT and MICHAEL L. NORRIS

Department of Molecular Embryology, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK

Summary

We have examined the role of germline-specific chromosomal determinants of development in the mouse. Studies were carried out using aggregation chimaeras between androgenetic ↔ fertilized embryos and compared with similar parthenogenetic ↔ fertilized chimaeras. Several adult chimaeras were found with parthenogenetic cells but none were found with androgenetic cells. Analysis of chimaeras at mid-gestation showed that parthenogenetic cells were detected in the embryo and yolk sac but that androgenetic cells were found only in the trophoblast and yolk sac and not in the embryo. The contribution of parthenogenetic cells to the embryo and yolk sac was increased by aggregating 2-cell parthenogenetic and 4-cell fertilized embryos but the contribution of parthenogenetic cells in extraembryonic tissues remained negligible even after aggregation of 4-cell parthenogenetic and 2-cell fertilized embryos. Furthermore, parthenogenetic cells were primarily found in the yolk sac mesoderm and not in the yolk sac endoderm.

These results suggest that maternal chromosomes in parthenogenetic cells permit their participation in the primitive ectoderm lineage but these cells are presumably eliminated by selective pressure or autonomous cell lethality from the primitive endoderm and trophoblast lineages. Conversely paternal chromosomes in androgenetic cells confer opposite properties since the embryonic cells can be detected in the trophoblast and the yolk sac but not in the embryos, presumably because they are eliminated from the primitive ectoderm lineage. The spatial distribution of cells with different parental chromosomes may occur partly because of differential expression of some genes, such as proto-oncogenes, and partly due to their ability to respond to a variety of diffusible growth factors.

Key words: parthenogenones, androgenones, chimaeras, parental chromosomes, genomic imprinting, mouse development, chromosomal determinants.

Introduction

Recent studies have shown that the parental origin of chromosomes influences both embryonic phenotype and the spatial distribution of cells during development (Surani *et al.* 1987). Maternal chromosomes are apparently required for development of the embryo while paternal chromosomes are essential for normal proliferation of extraembryonic tissues such as trophoblast (Surani *et al.* 1984; Barton *et al.* 1985; McGrath & Solter, 1984). Therefore, both parental genomes are needed for development to term (Surani *et al.* 1984; McGrath & Solter, 1984; Mann & Lovell-Badge, 1984; Renard & Babinet, 1986; Anderegg & Markert, 1986; Solter, 1987). Genetic studies have identified a number of autosomal regions whose functions are determined by their parental origin

(Cattanach & Kirk, 1985; Searle & Beechey, 1985; Cattanach, 1986). Differential expression of some parental alleles within these chromosomal domains may give rise to functional differences between parental genomes. Recent studies demonstrate germline-specific methylation or 'imprinting' of transgenes with a potential to influence their subsequent expression (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). Hence a molecular mechanism exists to distinguish between parental alleles and such modifications may serve as chromosomal determinants of development which can dictate the developmental potential of embryonic cells.

Experimental reconstruction of embryos with cells of different genotypes is one of the ways to examine the influence of the parental origin of chromosomes on development. The developmental potential of

embryonic cells can be examined in aggregation chimaeras between embryos of different genotypes (McLaren, 1976). Any deviation from the normal random distribution of embryonic cells in chimaeras would reflect the influence of parental genomes on their developmental capacity. Previous studies on chimaeras between normal and parthenogenetic embryos gave rise to adult chimaeras with a widespread contribution of parthenogenetic cells to somatic tissues and germ cells (Surani *et al.* 1977; Stevens *et al.* 1977; Stevens, 1978). In this study, we have examined the fate of androgenetic cells in a similar combination and compared it with more detailed studies on the developmental potential of parthenogenetic cells in aggregation chimaeras with fertilized embryos.

Materials and methods

Animals

CFLP albino outbred mice (AFRC colony from Bantin and Kingman stock) bred to be homozygous for the slow moving form of glucose phosphate isomerase (GPI) (Gpi-1^a/Gpi-1^a) and nonalbino (C57BL/6J×CBA/Ca)F₁ (from Bantin and Kingman stock, referred to as F₁) mice (Gpi-1^b/Gpi-1^b) were used in these experiments. 4- to 6-week-old females were injected with 7.5 i.u. pregnant mare's serum (Intervet Ltd, Cambridge) followed 42–48 h later by 7.5 i.u. human chorionic gonadotrophin (hCG) to induce superovulation (Fowler & Edwards, 1957).

Normal embryos

2-cell or 4-cell embryos were obtained following fertilization *in vivo* from F₁×F₁ or CFLP×CFLP matings. The embryos were retrieved either on day 1 or day 2 of pregnancy (day 1 = day of vaginal plug) and cultured *in vitro* in T6 (Howlett *et al.* 1987) + bovine serum albumin (BSA; Sigma) at 37.8°C in 5% CO₂ in air.

Parthenogenetic embryos

Unfertilized eggs from F₁ mice were obtained at 16.5–17.5 h post-hCG. The cumulus cells were removed by incubation with 300 i.u. ml⁻¹ hyaluronidase (ovine testis type V, Sigma) in PBI+BSA (Whittingham & Wales, 1969) for 3 min and washed twice with medium T6+BSA. The eggs were then activated in T6+BSA which contained 7% ethanol for 4.5 min at room temperature (Kaufman, 1982; Cuthbertson, 1983), washed six times with T6+BSA and cultured in this medium with 5 µg ml⁻¹ cytochalasin B (in 0.1% dimethylsulphoxide) for 3.5–4 h at 37.8°C in 5% CO₂ in air. The eggs were then washed nine times in T6 + BSA and cultured for a further period of 2 h. After this time, all the diploid eggs containing two pronuclei produced by the suppression of second polar body extrusion by cytochalasin B (Niemierko, 1975), were separated (60–90%) from haploid, fragmented and other abnormal eggs and cultured for 1–2 days in T6 + BSA.

Androgenetic embryos

Heterozygous diandric eggs were prepared as described previously (McGrath & Solter, 1983; Barton *et al.* 1984). Fertilized F₁♀ × CFLP♂ eggs were used as both recipient and donor eggs, the donor eggs being selected to be a little in advance of the host eggs (i.e. the pronuclei beginning to move towards the centre of the eggs). The pronuclear eggs were harvested approximately 18 h post HCG and the cumulus cells were removed as described above. Manipulations were carried out in PBI+BSA (Whittingham & Wales, 1969) with 1 µg ml⁻¹ cytochalasin D and 0.1 µg ml⁻¹ nocodazole.

The female F₁ pronucleus was removed from each of a group of the host eggs and a second male CFLP pronucleus from the donor group was introduced under the zonae of the now haploid host eggs, together with about 10 pl of inactivated Sendai virus to induce fusion of the karyoplast with the egg.

After washing and checking for fusion (about 95%) the reconstituted androgenetic eggs were cultured for 2 days in T6 + BSA. Only 4-cell embryos were used to make aggregation chimaeras.

Preparation of aggregation chimaeras and transfer to the uterus

Aggregation chimaeras were made using either 2-cell or 4-cell embryos as described below. The zona pellucida was first removed by a brief exposure (5–20 s) to acid Tyrode's medium, pH 2.0 (Nicolson *et al.* 1975) and washed six times in T6 + BSA. The two types of embryos to be aggregated were then placed in separate 20 µl drops of T6 + BSA medium under liquid paraffin oil. Each pair of embryos was aggregated by pushing the two embryos together until the aggregate seemed sufficiently stable. After all the pairs of aggregated embryos had been checked, they were cultured in T6 + BSA in 5% CO₂ in air. All aggregates were rechecked after 2–4 h to ensure that they had not drifted apart. The chimaeric embryos were then left in culture for 48 h if both embryos were at the 4-cell stage at the time of aggregation, or for 72 h if asynchronous embryos were aggregated in which one of the embryos was at the 2-cell stage. All the aggregated embryos that developed into morphologically normal blastocysts were transferred to day-3 pseudopregnant recipient F₁ females obtained by mating with vasectomized males of proven sterility. The recipients were either left until day 20 of gestation for development of the embryos to term or the fetuses were removed by autopsy on day 10 of gestation when the implantation sites were dissected to retrieve the conceptuses.

Dissection of day-10 embryos

The midgestation embryos were isolated from the implantation sites and carefully dissected to separate the embryos, visceral yolk sac and trophoblast. The embryos were staged and measured. The trophoblast was washed very thoroughly in several changes of PBI+BSA to reduce as far as possible contamination by maternal blood (GPI-1B) in the trophoblast. In all instances, F₁ recipients were used because they proved much more effective as host mothers

than the CFLP females. In some instances, the visceral yolk sac was treated further to separate the inner mesodermal layer from the outer endodermal layer. For this purpose, yolk sacs were placed in calcium-free DMEM medium containing 2.5% pancreatin + 0.5% trypsin + 0.5% polyvinylpyrrolidone (Gardner & Rossant, 1979) and kept in a refrigerator for 0.5–1.0 h. The yolk sacs were then washed in DMEM + 10% fetal calf serum and incubated in this medium for about 1 h. The endoderm and mesoderm could then be separated from each other under a dissecting microscope with the help of watchmaker's forceps. All the tissues were stored at -20°C until analysed for GPI.

Analysis for GPI was carried out on cellulose acetate using Helena Titan III electrophoresis plates as described previously (Eicher & Washburn, 1978). The proportion of GPI-1A and GPI-1B in each tissue was estimated by comparison with reference gels in which different proportions of GPI-1A and GPI-1B were analysed.

Results

Development of aggregation chimaeras to term

Of the 46 parthenogenetic $F_1 \leftrightarrow \text{CFLP} \times \text{CFLP}$ fertilized blastocysts transferred, 13 live young were born, 5 of which were chimaeric as judged by their coat colour (Table 1). The remaining animals were albino and when these animals were killed and their tissues checked by GPI typing none were found to be chimaeric. From a total of 96 morphologically normal blastocysts obtained after aggregation of 4-cell CFLP+CFLP androgenetic \leftrightarrow 4-cell $F_1 \times F_1$ fertilized embryos, 31 live young were born but all these were nonalbino with no evident contribution from androgenetic cells. All the adults were therefore killed and their tissues were analysed but in none of the tissues was there any evidence for cells with the GPI-1A isozyme which would have demonstrated contribution from androgenetic cells. A further experiment was therefore carried out in which 4-cell androgenetic embryos were aggregated with 2-cell fertilized embryos. This experiment was based on previous

Table 1. *Development of aggregation chimaeras using parthenogenetic or androgenetic embryos with fertilized embryos*

Type	No. of embryos* transferred	Number of live young	
		Total	Chimaeric
4-cell F \leftrightarrow 4-cell F	53	32	15
4-cell P \leftrightarrow 4-cell F	46	13	5
4-cell A \leftrightarrow 4-cell F	95	31	0
4-cell A \leftrightarrow 2-cell F	48	24	0

P, parthenogenetic embryos; A, androgenetic embryos; F, fertilized embryos.

* Morphologically normal blastocysts were selected and transferred to day-3 pseudopregnant recipients.

findings which showed that the more advanced embryos contribute a greater proportion of cells to the embryo while the earlier stage embryos contribute a greater proportion of cells to the extraembryonic lineage (Kelly *et al.* 1978; Surani & Barton, 1984). Such asynchronous aggregations were therefore carried out to increase the chances of contribution of androgenetic cells to the embryo itself. However, of the 24 animals born from such aggregated embryos, none showed coat colour chimaerism nor could the cellular contribution from androgenetic cells to any of the tissues be detected by analysis for GPI isozymes. Hence, only parthenogenetic embryos appeared to contribute cells to chimaeric adults whilst androgenetic cells could not be detected in any of the adult animals obtained from aggregation chimaeras.

Distribution of androgenetic and parthenogenetic cells in chimaeric fetuses at midgestation

Since no androgenetic cells were detected in adults, experiments were carried out to determine the distribution of parthenogenetic and androgenetic cells in fetuses at midgestation. Aggregation chimaeras between 4-cell androgenetic and 4-cell fertilized embryos were analysed on day 10 of gestation in most cases and compared with similar chimaeras between parthenogenetic and fertilized embryos (Fig. 1). In these studies, androgenetic cells were detected in the midgestation embryos but they were confined to the trophoblast and the yolk sac where in some cases they made up more than 50% of the tissue (Fig. 1A). It was only in one case that androgenetic cells were detected in the embryo itself and this embryo was degenerating at the time of analysis. The distribution of parthenogenetic cells in midgestation chimaeric fetuses was the opposite to that observed for androgenetic cells (Fig. 1B). Parthenogenetic cells were found predominantly in the embryo where they contributed over 45% of the cells and in the yolk sac where overall they represented nearly 35% of the tissue (Fig. 2). The proportion of parthenogenetic cells present in the trophoblast was negligible and this was on average only about 10%. This pattern of distribution of cells is consistent with the finding that only parthenogenetic cells could be detected in adults because androgenetic cells are confined to the extraembryonic tissues.

Further analysis of the distribution of parthenogenetic cells in midgestation embryos

Detailed investigations of the influence of parental origin of chromosomes on spatial distribution of embryonic cells was carried out using parthenogenetic embryos, since androgenetic eggs are laborious to prepare and only rarely do more than 10% of them reach the blastocyst stage in culture. The purpose of

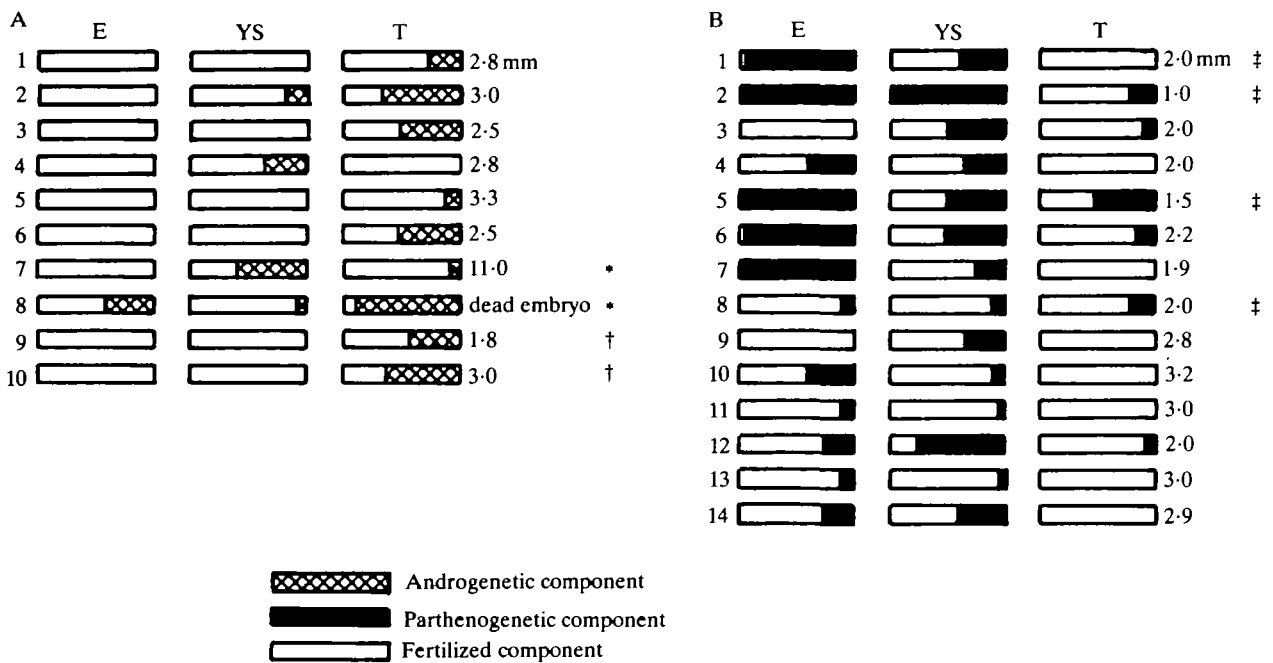


Fig. 1. Bar charts of the proportion and gross distribution of androgenetic (A) and parthenogenetic (B) cells in synchronous chimaeras made by aggregation with fertilized embryos (4-cell with 4-cell) analysed at midgestation by GPI typing.

†, day-11 embryos; *, day-14 embryos; all the rest are day-10 embryos.

‡, Trophoblast very slight.

E, embryo; YS, yolk sac; T, trophoblast.

The androgenetic series includes results obtained before we determined that day 10 is the most informative day for analysis. The 10 chimaeras presented are the product of over 1000 androgenetic eggs constructed.

Nonchimaeric conceptuses (29 in the androgenetic series, 1 in the parthenogenetic series) have been excluded.

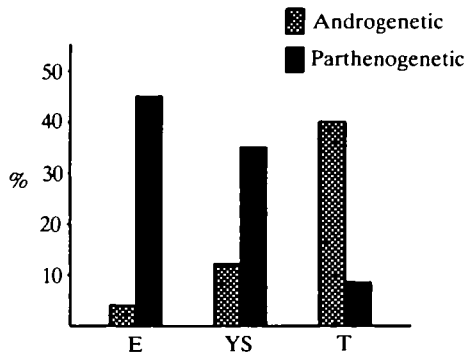


Fig. 2. Histogram of the proportion and distribution of parthenogenetic and androgenetic cells in the synchronous chimaeras with normal embryos presented in Fig. 1.

these studies was firstly to determine whether parthenogenetic cells could be made to contribute to the trophoblast tissue. Second, the relative proportion of parthenogenetic cells in the yolk sac mesoderm was compared with that in the yolk sac endoderm.

In aggregation chimaeras between 4-cell fertilized and 4-cell parthenogenetic embryos, both cell types were present in almost equal proportions in the embryo but the number of parthenogenetic cells in

the trophoblast was negligible (Figs 3A, 4). Analysis of the yolk sac revealed that the parthenogenetic cells were present to a far greater extent in the yolk sac mesoderm than in the yolk sac endoderm. In asynchronous aggregation chimaeras between 4-cell parthenogenetic and 2-cell fertilized embryos, the proportion of parthenogenetic cells in embryos increased substantially to 60% and in the yolk sac mesoderm to over 60%. However, the contribution of parthenogenetic cells to the yolk sac endoderm and trophoblast remained below 10% (Figs 3B, 4). When such aggregation chimaeras are allowed to develop to term, a far larger number of chimaeras are obtained with a substantial contribution to adult tissue from parthenogenetic cells (D. G. Whittingham, unpublished data; R. Fundele, unpublished data). By contrast, 4-cell androgenetic embryos aggregated with 2-cell fertilized embryos failed to produce any chimaeric adults with androgenetic cells.

Further studies were carried out with aggregation of 2-cell parthenogenetic embryos with 4-cell fertilized embryos. This was an attempt to shift the balance of parthenogenetic cells from embryonic to extraembryonic tissues. As expected, the contribution of parthenogenetic cells to the embryo was

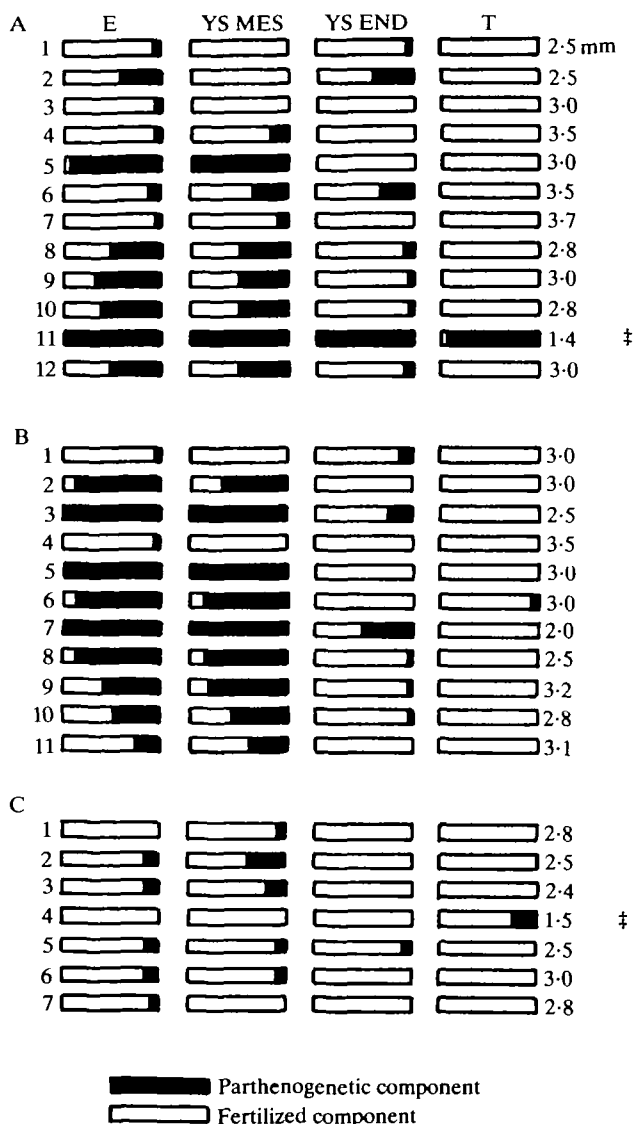


Fig. 3. Bar charts of the proportion and distribution of parthenogenetic cells in chimaeras with fertilized embryos in which the yolk sac was separated into the mesoderm and endoderm layers, analysed at day 10 of gestation. (A) Synchronous aggregations, 4-cell with 4-cell. (B) Asynchronous aggregations, 4-cell parthenogenones with 2-cell fertilized embryos. (C) Asynchronous aggregations, 2-cell parthenogenones with 4-cell fertilized embryos. ‡, Trophoblast very slight; E, embryo; YS MES, yolk sac mesoderm; YS END, yolk sac endoderm; T, trophoblast. All the embryos were 25 somite or more, with the exception of C7 which was an unturned embryo of about 12 somites; the trophoblast in this conceptus was very slight. Nonchimaeric all-fertilized conceptuses were excluded from all series: four from A, three from B and three from C.

reduced to less than 10%, as was also the case in the yolk sac mesoderm. However, there was no concomitant rise in the proportion of parthenogenetic cells in the yolk sac endoderm and trophoblast, which

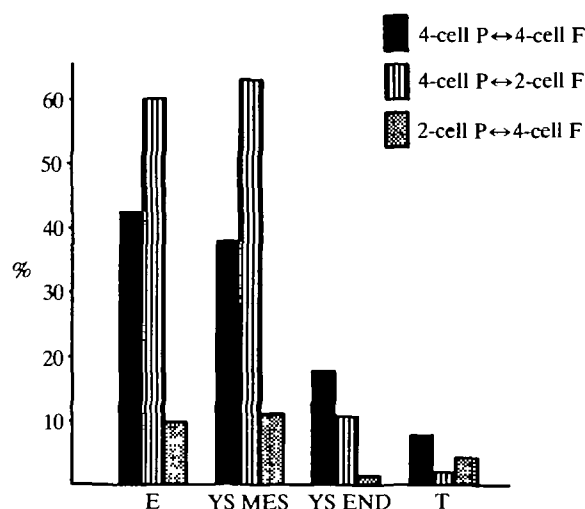


Fig. 4. Histogram of the proportion of parthenogenetic cells in the midgestation synchronous and asynchronous chimaeras with fertilized embryos presented in Fig. 3.

remained at less than 5% (Figs 3C, 4). Hence the contribution by androgenetic cells especially to the trophoblast of over 30% in 4-cell-fertilized ↔ 4-cell-androgenetic embryos was far greater than could be achieved with parthenogenetic embryos despite efforts to increase the proportion of parthenogenetic cells in the trophoblast.

Discussion

This study on aggregation chimaeras with fertilized embryos demonstrated that the pattern of distribution of androgenetic cells is the reverse of that seen with parthenogenetic cells which survive into adulthood and contribute to all somatic tissues and germ cells (Surani *et al.* 1977; Stevens *et al.* 1977; Stevens, 1978). By contrast, androgenetic cells could not be detected in any of the live young examined even when more advanced 4-cell androgenetic embryos were combined with 2-cell fertilized embryos, although the more advanced embryo has the potential to contribute a larger proportion of cells to the resulting embryo (Kelly *et al.* 1978; Surani & Barton, 1984).

Androgenetic cells were detected in chimaeric fetuses at midgestation but they were confined to the trophoblast and yolk sac while the parthenogenetic cells were found in the embryo and yolk sac. A similar distribution of cells was previously observed in androgenetic ↔ parthenogenetic fetuses (Surani *et al.* 1987). This spatial distribution of cells is a reflection of their lineage specificity; parthenogenetic cells were found predominantly in the yolk sac mesoderm and to a lesser extent in the yolk sac endoderm suggesting that embryonic cells with only maternal chromosomes are better able to survive in the primitive

ectoderm lineage. The developmental potential of parthenogenetic cells was tested further in aggregation chimaeras using asynchronous embryos to determine if the proportion of parthenogenetic cells in different lineages could be altered, more especially in the trophoblast and primitive endoderm lineages. In these studies, the contribution of parthenogenetic cells to the trophoblast and yolk sac endoderm did not increase even in asynchronous chimaeras between 2-cell parthenogenetic ↔ 4-cell fertilized embryos which demonstrates that even the less advanced parthenogenetic cells show little propensity for contribution to the trophoblast and yolk sac endoderm. Interestingly, the contribution of parthenogenetic cells to the yolk sac mesoderm and embryos did on the other hand increase substantially in the reciprocal aggregation chimaeras between 2-cell-fertilized ↔ 4-cell-parthenogenetic embryos. Therefore, cells with maternal chromosomes can participate in the development of tissues derived from primitive ectoderm and survive to contribute to somatic tissues and germ cells in adults while those that contain paternal chromosomes are confined to the trophoblast and yolk sac.

It would be interesting to examine the fate of gynogenetic cells in chimaeras. The incidence of chimaerism with uniparental homozygous gynogenetic embryos was exceedingly low (Anderegg & Markert, 1986). Perhaps the low frequency of chimaerism can be partly attributed to the fact that, in these gynogenones, the first cleavage division has to be suppressed by relatively prolonged incubation of the eggs in cytoskeletal inhibitors to restore diploidy. There is also a greater likelihood of the expression of recessive lethal genes. In our experiments on reconstitution of blastocysts from the inner cell mass and trophoblast, there was no detectable difference in the developmental potential between the parthenogenetic and heterozygous biparental gynogenetic tissues (Barton *et al.* 1985). Hence it is likely that the latter will produce similar results to those reported here using parthenogenetic embryos.

Whether specific embryonic cells survive or are selected against in the presence of normal cells may depend partly on their inherent developmental potential and partly on their ability to respond to cell interactions, gap junctional communications or to a number of diffusible factors. Selection pressure is suggested to occur against parthenogenetic cells in the yolk sac and in a more protracted fashion in the embryo as well (Nagy *et al.* 1987). This is despite the fact that, in the absence of a paternal X-chromosome in the parthenogenetic yolk sac, one of the two maternally derived X-chromosomes is inactivated (Rastan *et al.* 1980). Nevertheless, prolonged survival of parthenogenetic cells occurs in the presence of

normal cells both in fetal and adult chimaeras as well as in extrauterine ectopic sites (Iles *et al.* 1975). In both instances, the extensive proliferation and differentiation of parthenogenetic cells observed could be due to the availability of a variety of diffusible growth factors produced by normal tissues. In this respect, the differential expression of a number of proto-oncogenes encoding growth factors and receptors in the embryonic and extraembryonic tissues is probably important (reviewed by Adamson, 1987) both in normal development and in explaining the behaviour of parthenogenetic and androgenetic cells. There is, for example, evidence to suggest that autocrine factors may be implicated in the control of cell proliferation in the human cytotrophoblast where elevated expression of *c-myc* and *c-fos* and increased DNA synthesis may result directly from expression of *c-sis* (Goustin *et al.* 1985) which encodes a protein with PDGF-like activity (Waterfield *et al.* 1983). The parental origin of chromosomes may dictate expression of complementary genes involved in the response of cells to such extracellular signals for cell proliferation and differentiation. This could also explain why androgenetic ↔ parthenogenetic chimaeras fail to develop to term because of the absence of normal cells.

Any mechanisms such as the one discussed above that is responsible for the control of spatial distribution and developmental potential of embryonic cells has ultimately to be explained in terms of the influence of chromosomal determinants of development. The proposed reversible germline-specific modifications or 'imprinting' of some chromosomal domains could be responsible for differential roles of parental genomes (Surani *et al.* 1986). From genetic studies (Cattanach & Kirk, 1985; Searle & Beechey, 1985; Cattanach, 1986) and those involving observations on transgenes (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987), both the functional differences between some parental chromosomal domains and a molecular mechanism to distinguish between transgene parental alleles have been demonstrated. The modifications and differential expression of certain endogenous genes that depend on their parental origin could underly the observed spatial distribution of androgenetic and parthenogenetic cells. None of the endogenous genes which may serve this type of function have yet been identified. Whether these are specific housekeeping genes or whether they represent a category of regulatory 'imprinted' genes serving a higher order function affecting expression of other genes, such as proto-oncogenes, also remains to be established. These studies on the growth, differentiation and spatial distribution of embryonic cells could prove significant for understanding the mechanism that underlies aberrant cellular growth of the

human syncytiotrophoblast in androgenetic hydatidiform moles (Bagshawe & Lawler, 1982; Szulman & Surti, 1982) and more generally of the unrestricted growth of malignant cells in adults.

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