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Nucleocytoplasmic interactions in the mouse embryo

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INTRODUCTION

Fertilized mammalian ova consist of haploid genomes derived from both parents and cytoplasmic components inherited largely from the female parent. These three cellular compartments must successfully interact with each other and with their environment for development to proceed. These interactions require the transposition of nuclear and cytoplasmic products between cellular compartments with resultant alteration of gene transcription and the cytoplasmic expression of preformed or newly synthesized gene products. We have investigated nuclear/cytoplasmic interactions in the mouse embryo *via* the microsurgical transfer of nuclei and cytoplasm. Experiments have specifically examined the ability of nuclei from later developmental stages or from a different species to support development, volume relationships between nuclear and cytoplasmic compartments, and the nonequivalency of the maternal and paternal genomic contributions to development.

The ability of egg cytoplasm to alter the function of a variety of embryonic and adult nuclei and the ability of these nuclei to support development has been extensively tested in nuclear transplantation investigations in amphibian embryos. These studies have shown that (a) early embryonic nuclei can support complete development (Briggs & King, 1952), (b) nuclei from progressively later developmental stages are less able to support development with serial transfer nuclei undergoing characteristic, clone-specific, developmental arrest (King & Briggs, 1956; Subtelny, 1965), and (c) instances of extensive but incomplete development have been achieved with differentiated adult nuclei (Gurdon, 1962; Gurdon & Uehlinger, 1966; Laskey & Gurdon, 1970; Gurdon & Laskey, 1970).

The inability of differentiated nuclei to support complete development has been explained alternatively by the irreversibility of differentiation or the inability of the transplanted nuclei to revert to the rapidly dividing cleavage state without sustaining lethal chromosomal damage. Evidence for the latter is supported by cytological studies of amphibian nuclear transplant embryos (Briggs, Signoret & Humphrey, 1964; DiBerardino & Hoffner, 1970).

Key words: nucleocytoplasmic interactions, mouse embryo transplantation, nuclear transfer, interspecific, haploid, T^{hp} mutation, parthenogenones

NUCLEAR TRANSPLANTATION IN MOUSE EMBRYOS

Initial attempts to introduce nuclei into mammalian embryos utilized sendai virus to fuse somatic cells with oocytes or early-cleavage-stage embryos (Graham, 1971; Lin, Florence & Oh, 1973; Baranska & Koprowski, 1970). Evidence that foreign nuclei could persist in the mammalian embryo was achieved when Modliński (1978) injected 8-cell nuclei with the T6 marker chromosome into nonenucleated zygotes and demonstrated the presence of the marker chromosome in resultant tetraploid blastocysts. In a subsequent investigation Modliński (1981) demonstrated the similar ability of inner cell mass (ICM) cell, but not trophectoderm, nuclei to contribute to tetraploid blastocysts when injected into nonenucleated zygotes.

A more rigorous test of the ability of foreign nuclei to support development is their placement in enucleated cytoplasm. Although the removal of the zygote pronuclei can be accomplished *via* micropipette penetration of the ovum plasma membrane (Modliński, 1975, 1980; Markert & Petters, 1977; Hoppe & Illmensee, 1977; Illmensee & Hoppe, 1981), these embryos frequently disintegrate following manipulation. The development of a karyoplast fusion method in which nuclei can be removed or introduced into mouse embryos without cell membrane disruption (McGrath & Solter, 1983*a*) has greatly facilitated mammalian nuclear transplantation studies. In our initial description of this technique, 69 pronuclei were reciprocally transplanted between zygotes of mouse strains that differed in their coat colour phenotype. After transfer of resultant blastocysts into pseudopregnant females, ten progeny with the coat colour phenotype of the donor nuclei resulted, seven of which survived to adulthood. This result compared favourably with the number of progeny born to control embryos (5/34).

Having established that the nuclear transfer method was well tolerated by the embryo, we tested nuclei from successive preimplantation stages (2-, 4-, 8-cell embryos and ICM cells) for their ability to support *in vitro* development when transplanted into enucleated zygotes (McGrath & Solter, 1983*b*, 1984*a*). Our results showed that whereas 95% of enucleated zygotes receiving pronuclei developed to the morula–blastocyst stages, only 19% of enucleated zygotes receiving 2-cell-stage nuclei did so. Complete preimplantation development of enucleated zygotes receiving 8-cell or ICM nuclei was not observed.

In the preceding experimental series, however, nuclear introduction occurred during the *latter* half of the first cleavage division. Developmental failure of these nuclear transfer embryos, therefore, may have resulted from an inadequate exposure of the donor nuclei to cytoplasmic signals present for only a short period of time following activation. It is interesting to note that Czołowska, Modliński & Tarkowski (1984) observed the swelling of thymocyte nuclei in activated ovum cytoplasm to equal that of pronuclei when nuclear introduction coincided with activation, but diminished with increasing time between activation and nuclear introduction.

In order to permit earlier exposure of donor nuclei to activated ovum cytoplasm, we therefore introduced 8- to 16-cell mouse embryo nuclear karyoplasts into activated oocytes within 3 h of activation. Nuclear introduction was accomplished using either inactivated sendai virus or electrofusion (Kubiak & Tarkowski, 1985). 4–6 h after activation the newly visible maternal pronuclei were microsurgically removed and the embryos permitted to develop *in vitro*. Our results (Table 1) show that 74% of control activated oocytes receiving two zygote pronuclei developed to the morula–blastocyst stages. In contrast, of 198 activated oocytes receiving 8- to 16-cell-stage nuclei, 112 (57%) never divided and 61 (31%) divided only once. Thus, despite the presence of these nuclei in the ovum cytoplasm for an extended period of time, very few nuclear transplant embryos were able to develop. Nevertheless, a small proportion (3%) of the manipulated embryos did achieve the 8-cell, morula and blastocyst stages suggesting that in exceptional instances complete preimplantation development may be supported by mid-cleavage nuclei. However, caution must be exercised in interpreting this result since the donor origin of these nuclei was not confirmed. Future experiments will attempt to demonstrate nuclear origin and define parameters that may increase the frequency of successful nuclear transplant embryo development. A possible approach to the latter would be to extend the time that donor nuclei reside in early ovum cytoplasm by serially transplanting nuclei into activated oocytes on successive days. The adaptation of electrofusion to the mammalian embryo (Kubiak & Takrowski, 1985), which should permit serial karyoplast fusions (not readily performed with sendai virus-mediated fusions; McGrath & Solter, unpublished observation), could facilitate such an investigation. Preliminary evidence demonstrating the ability to passage nuclei through several mouse embryo first cell cycles has been obtained (McGrath & Solter, unpublished observations).

Recently, the fusion of 8-cell-stage sheep embryo blastomeres with half of the cytoplasmic volume of activated oocytes has led to the birth of live progeny (Willadsen, 1986). However, Willadsen (1981) had previously demonstrated that single 8-cell-stage sheep embryo blastomeres without the addition of activated ovum cytoplasm will, at a reduced frequency, also give rise to live progeny. Therefore, whether the addition of activated ovum cytoplasm to single 8-cell blastomeres results in significant nuclear reprogramming remains unanswered. Nevertheless, these experiments underscore significant species differences between mammalian embryos. It is of interest to note that ultrastructural changes of nucleoli consistent with active rRNA synthesis occurs in the mouse embryo at the late 2-cell stage (Hillman & Tasca, 1969) but is not observed in sheep embryos until the 16-cell stage (Calarco & McLaren, 1976). In addition, whereas single 8-cell-stage sheep embryo blastomeres will give rise to live progeny (Willadsen, 1981), single 8-cell-stage mouse embryo blastomeres do not complete development (Tarkowski & Wroblewska, 1967; Rossant, 1976), unless combined with additional blastomeres (Kelly, 1975).

Table 1. *Ability of 8- to 16-cell-stage blastomere nuclei to support development when transferred into enucleated activated mouse oocytes*

	Total	1-cell	2-cell	3-cell	4-cell	8-cell	Morula	Blastocyst
8- to 16-cell nuclei	198	112 (57)*	61 (31)	13 (7)	6 (3)	4 (2)	1 (0·5)	1 (0·5)
Zygote pronuclei	24	2 (8)	2 (8)	0	2 (8)	0	2 (8)	16 (67)

* Total number of embryos achieving developmental stage after 5 days of *in vitro* culture.

Oocytes were obtained from C57B16/J females that received an intraperitoneal injection of 5 i.u. pregnant mares serum gonadotropin followed 48 h later by an injection of 5 i.u. human chorionic gonadotropin (HCG). 16 h post-HCG injection, females were sacrificed and oocytes were removed from the ampullary regions of excised oviducts. Cumulus cells were removed by a brief incubation in Whitten's medium which contained 500 units bovine hyaluronidase (Sigma). Oocytes were washed in Hepes-buffered Whitten's medium (HWM) and activated by a 7 min incubation in 7 % ethanol in HWM at room temperature (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982).

Enucleation and the placement of pronuclear and 8- to 16-cell nuclear karyoplasts into the perivitelline space of oocytes was performed as previously described (McGrath & Solter, 1983*a*,*b*). Karyoplasts were fused with oocytes using either inactivated sendai virus (McGrath & Solter, 1983*a*) or electrofusion (Reichert, Scheurich & Zimmerman, 1981) as modified for the mouse embryo by Kubiak & Tarkowski (1985). Electrofusion was performed on a dissecting microscope with a pulse generator (Grass medical instruments) which delivered two 25 V pulses of 100 μs duration. At 4–6 h after activation the maternal pronucleus was removed from successfully fused karyoplast: oocyte pairs.

In preliminary experiments in which 8- to 16-cell-stage nuclei were randomly introduced into activated oocytes prior to second polar body extrusion, difficulty was encountered in distinguishing the donor 8- to 16-cell-stage nuclei from the maternal pronucleus. Therefore, activated oocytes were cultured *in vitro* and were used as recipients only after second polar body extrusion had occurred (45 min following activation). Nuclei were introduced no more than 3 h after activation of oocytes. Introduction of the donor nuclei opposite the site of second polar body extrusion permitted easy identification of the donor and maternal nuclei. Following microsurgery, embryos were washed and cultured for 5 days in Whitten's medium (Whitten, 1971) as modified by Abramczuk, Solter & Koprowski (1977) under silicone oil in an atmosphere of 5 % O_2, 5 % CO_2 and 90 % N_2.

INTERSPECIFIC NUCLEAR TRANSFERS

Preliminary results of transfers of both pronuclei between *Mus musculus* and *Mus caroli* zygotes revealed developmental arrest of these embryos at or prior to the 4-cell stage (Solter, Aronson, Gilbert & McGrath, 1985). The inability of these interspecific nuclear transplant embryos to develop does not result from the simultaneous transfer of membrane and/or cytoplasm in the pronuclear karyoplast since control embryos that received only interspecific membrane-bound cytoplasm can develop to term (McGrath & Solter, unpublished observations). These preliminary data indicate that early embryonic development is dependent upon correct nuclear/cytoplasmic interactions that may not efficiently operate between disparate species. Future experiments will hopefully expand the number and variety of interspecific transfers and examine the ability of such embryos to develop when subjected to serial nuclear transplantations.

NUCLEAR/CYTOPLASMIC RATIOS: HAPLOID EMBRYO DEVELOPMENT

The above data demonstrate that in the mouse embryo successful development is dependent upon nuclear/cytoplasmic compatibility. To determine whether nuclear/cytoplasmic components exhibit quantitative interactions, we examined the effect of the nuclear/cytoplasmic (N/C) ratio on haploid embryo development. Previous data have demonstrated that haploid embryos produced either by ovum activation (Witkowska, 1973; Kaufman & Gardner, 1974; Kaufman & Sachs, 1976) or by the microsurgical removal of a single pronucleus from fertilized zygotes (Modliński, 1975) develop poorly. Possible causes for this decreased development include the expression of recessive mutations, decreased heterozygosity or an altered nuclear/cytoplasmic ratio (Kaufman & Sachs, 1976). We have compared the developmental ability of haploid embryos produced by the removal of a single male or female pronucleus to haploid embryos that underwent normalization of their N/C ratio by removing a single pronucleus and additionally half the cytoplasmic volume of the zygote. Our results (Table 2) show that an increased proportion of these cytoplasm-depleted haploid embryos developed to the morula–blastocyst stages and therefore a normalization of the N/C ratio in haploid embryos results in improved, but not completely restored, development. This result is consistent with the previous observation in which an increased proportion of parthenogenetic haploid embryos that underwent immediate cleavage (and thus normalized their N/C ratio) developed beyond the 4-cell stage when compared to haploid embryos that possessed a decreased N/C ratio (Kaufman & Sachs, 1976). In the latter investigation, however, the authors concluded that the improved development of immediate cleavage embryos resulted from the increased heterozygosity that occurs in immediate cleavage embryos. We suggest improved development of these embryos occurs as a result of a more normal N/C ratio. It is of interest to note that a greater proportion of haploid embryos produced by zygote bisection (Tarkowski & Rossant, 1976;

Tarkowski, 1977) complete preimplantation development than haploid embryos produced by pronuclear removal alone (Modliński, 1975, 1980).

NONEQUIVALENCY OF THE MATERNAL AND PATERNAL GENOMES

Mammalian parthenogenones are inviable and generally undergo developmental arrest shortly after implantation. Development to the 25-somite stage has, however, been observed (Kaufman, Barton & Surani, 1977). Generalized cell lethality is not the cause of parthenogenetic inviability since parthenogenetic contributions to the adult soma and germline are observed in parthenogenetic–wild-type chimaeras (Stevens, Varnum & Eicher, 1977; Surani, Barton & Kaufman, 1977; Stevens, 1978). Proposed reasons for the death of parthenogenones have included the lack of an essential non-nuclear contribution by the spermatozoan, the inability of the activating stimulus to recreate the stimulus provided at fertilization, or homozygosity for recessive lethal mutations (Graham, 1974).

We and others have recently utilized nuclear transplantation to determine if parthenogenetic inviability might result from differential functioning of the maternal and paternal genomes during embryogenesis. In this proposal parthenogenetic embryos are inviable since they lack a paternal pronucleus, which, it is suggested, possesses unique functions not shared by the maternal pronucleus. Evidence that supports this proposal is summarized below.

Table 2. *The effect of reduced cytoplasmic volume on the ability of haploid androgenetic and gynogenetic embryos to develop* in vitro

	Total	1- to 3-cell	4- to 6-cell	8-cell	Morula	Blastocyst
Gynogenone	75	52 (69)*	9 (12)	3 (4)	3 (4)	8 (11)
Androgenone	74	48 (65)	16 (22)	9 (12)	0	1 (1)
Gynogenone–half cytoplasm	57	10 (18)	16 (28)	8 (14)	6 (11)	17 (30)
Androgenone–half cytoplasm	61	18 (30)	14 (23)	16 (26)	11 (18)	2 (3)
Unmanipulated control	105	0	2 (2)	2 (2)	10 (10)	91 (87)

* Furthest developmental stage achieved after 5 days of *in vitro* culture (%).

Fertilized zygotes were obtained from spontaneous C57B16/J *inter se* matings. Embryo isolation and pronuclear removal were as previously described (McGrath & Solter, 1983*a*, 1984*c*). Removal of one half the cytoplasmic volume of the zygote was accomplished in a manner essentially identical to that employed for pronuclear removal. Estimation of the volume of cytoplasm removed was guided by the use of an ocular micrometer. Preliminary experiments revealed that in some instances the mechanical stresses needed to remove half of the zygote cytoplasmic volume resulted in the 'fusion' of the second polar body with the zygote. Therefore, in all embryos in which the cytoplasmic volume was halved, the second polar body was microsurgically removed prior to cytoplasm removal. Following microsurgery, embryos were cultured *in vitro* as described in the legend to Table 1.

Nuclear transplantation of the T^{hp} mutation

Inheritance of the T^{hp} mutation, a deletion of the proximal portion of chromosome 17 in the mouse (Bennett *et al.* 1975; Silver, Artzt & Bennett, 1979), results in viable progeny when inherited from the male parent, whereas inheritance of this same mutation from the female parent results in embryonic lethality during the latter half of embryogenesis (gestational days 15–21) or shortly after birth (Johnson, 1974, 1975). We investigated the nuclear/cytoplasmic origin of maternal-T^{hp} lethality by performing reciprocal pronuclear transplantations between maternal-T^{hp} and +/+ zygotes (McGrath & Solter, 1984*b*). Our results showed that of 197 enucleated zygotes from T^{hp}/+ females receiving +/+ pronuclei, 45 normal-tailed progeny resulted. In contrast, of 206 +/+ enucleated zygotes receiving equal numbers of +/+ and T^{hp}/+ pronuclei, 16 normal-tailed and 2 short-tailed progeny resulted. Both short-tailed T^{hp}/+ progeny died within 24 h of parturition. We concluded that maternal-T^{hp} lethality is nuclear in origin and suggested differential functioning of the proximal portion of chromosome 17 during male *versus* female gametogenesis in the mouse.

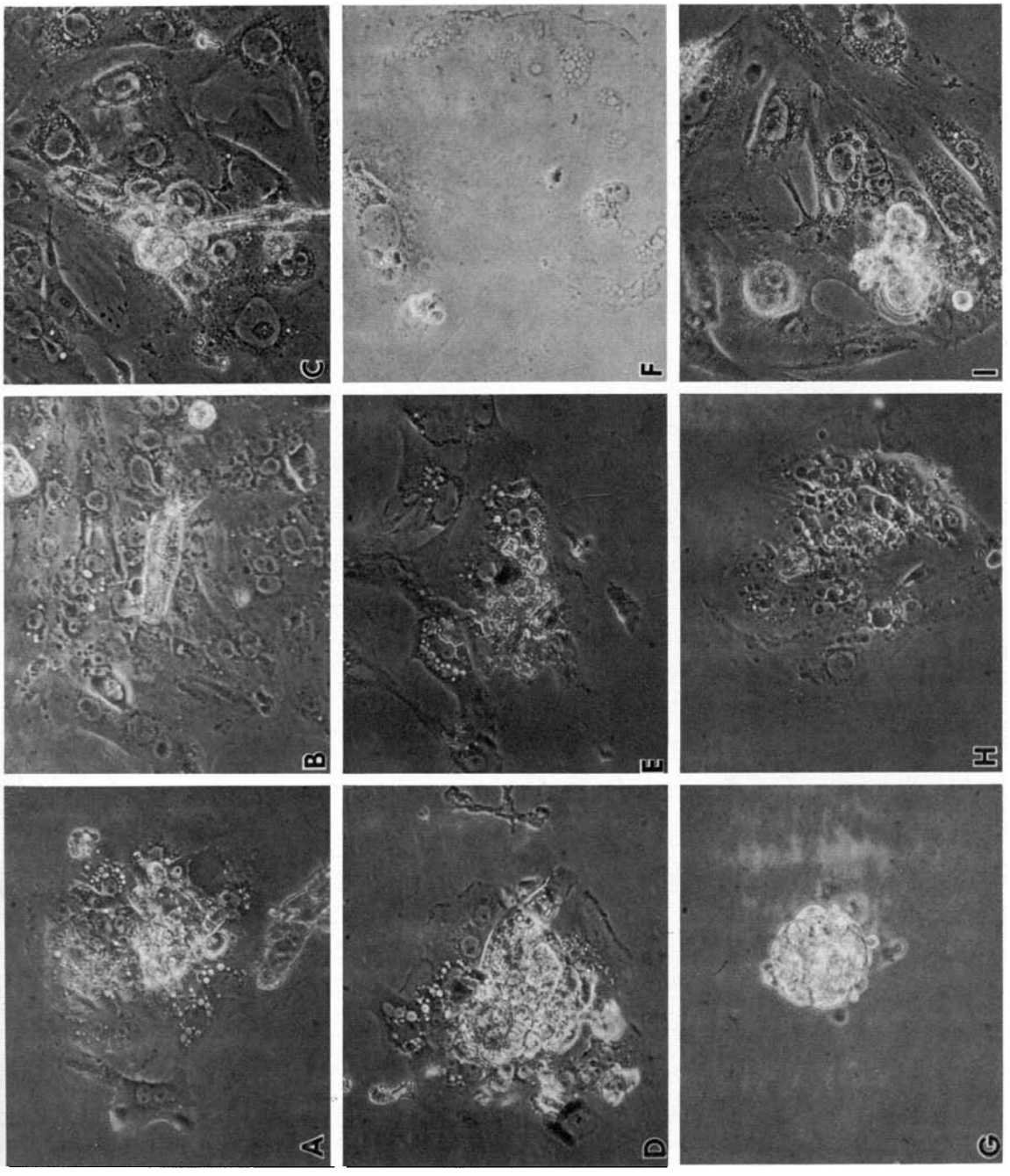
Nuclear transfers between parthenogenones and fertilized embryos

Parthenogenone inviability has been investigated by Surani, Barton & Norris (1984) by alternately introducing single male or female pronuclei into unfertilized activated oocytes possessing a haploid maternal genome. These investigators demonstrated that the introduction of a single male pronucleus restored complete development to haploid parthenogenones whereas the similar addition of a single female pronucleus resulted in early postimplantation lethality. In a similar investigation Mann & Lovell-Badge (1984) interchanged two maternal pronuclei from diploid parthenogenones with the male and female pronuclei from fertilized zygotes. Their results similarly showed that parthenogenetically activated cytoplasm could support complete development if it received a male and female pronucleus but that fertilized zygote cytoplasm receiving two female pronuclei underwent early postimplantation developmental arrest. These results demonstrate that parthenogenetic lethality does not result from a primary cytoplasmic deficiency.

Fertilized androgenetic and gynogenetic embryos

Fertilized diploid gynogenetic embryos have been produced by suppression of second polar body extrusion in fertilized embryos and the subsequent return of these triploid embryos to the diploid state *via* the microsurgical removal of the paternal pronucleus (Surani & Barton, 1983). Transfer of these gynogenones into pseudopregnant females resulted in early postimplantation lethality and thus inferred that parthenogenetic lethality does not result from a cytoplasmic deficiency.

We have also investigated the ability of gynogenetic embryos to develop by transplanting single pronuclei between fertilized zygotes. This experimental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design precludes any adverse effects of second polar body suppression and also permits the formation of androgenetic nuclear transplant embryos (McGrath & Solter, 1984*c*). Manipulated embryos were transferred into pseudopregnant females and permitted to develop to term. Three progeny were obtained from 339 gynogenetic embryos and two progeny were obtained from 328 androgenetic embryos. The phenotype of these five progeny, however, demonstrated that they all possessed a maternal/paternal origin and thus resulted from the incorrect assignment of the parental origin of the pronuclei at the time of nuclear transplantation. In contrast, 18 progeny were obtained from 348 control nuclear transplant embryos, in which a pronucleus was removed and replaced with a pronucleus of identical parental origin, all of whom demonstrated a maternal and paternal phenotype. A similar investigation (Barton, Surani & Norris, 1984) also produced androgenetic, gynogenetic and control nuclear transplant embryos. In this study, control embryos were similarly observed to develop to term whereas gynogenetic and androgenetic embryos were observed to exhibit marked growth retardation and malformations during early postimplantation development. These authors additionally noted that gynogenetic embryos demonstrated a deficiency of extraembryonic tissues. In contrast, androgenetic embryos possessed relatively intact extraembryonic membranes but incurred a disproportionate retardation in the development of embryonic structures.

In addition to nuclear transplantation investigations, two additional systems have demonstrated differential functioning of the maternal and paternal genomes during embryogenesis. The paternal X chromosome has been shown to undergo preferential X-inactivation in murine extraembryonic tissues (Takagi & Sasaki, 1975; West, Frels, Chapman & Papaioannou, 1977; Harper, Fosten & Monk, 1982). Additionally, genetic analysis of the products of meiotic adjacent-2 disjunction in the mouse have revealed functional differences in the maternal/paternal contributions to the embryonic genome (Lyon & Glenister, 1977; Searle & Beechey, 1978; Cattanach & Kirk, 1985). In the latter investigations translocation heterozygotes were utilized to generate gametes that possessed a parental duplication or deficiency of a single chromosome or chromosomal region. The union of two gametes possessing complementary duplications/deficiencies results in euploid embryos which inherit both copies of a chromosome or chromosomal region from a single parent. These studies have mapped specific regions of the genome that result in embryonic lethality or a distinct phenotype when inherited solely from one parent. In such an investigation Cattanach & Kirk (1985) have recently demonstrated that mice that inherit both their 11th chromosomes from their maternal parent have a decreased body size whereas mice that inherit this chromosome solely from their paternal parent possess an increased body size. The

Fig. 1. *In vitro* outgrowth of control (A–C), gynogenetic (D–F) and androgenetic (G–I) blastocysts on the second (left), fourth (middle) and sixth (right) day following transfer of blastocysts to Dulbecco's modified Eagle's medium. Embryos were observed using a Zeiss inverted-phase-contrast microscope.

demonstration that this opposite phenotypic difference persists into adulthood is of particular interest.

The preceding evidence demonstrates that functional differences between the maternal and paternal gametic genomes can result in phenotypic differences in early postimplantation embryos (Barton *et al.* 1984), gestational day 15–21 embryos (McGrath & Solter, 1984*b*) and adult mice (Cattanach & Kirk, 1985). We have attempted to determine whether such differences can be detected during preimplantation development or *in vitro* postimplantation development by comparing the ability of androgenetic, gynogenetic and control nuclear transplant embryos to develop *in vitro*. Of 69 gynogenetic embryos, 58 (84%) developed to the blastocyst stage whereas only 29 of 69 androgenetic embryos (42%) reached this developmental stage. The decreased development of androgenetic embryos would appear to be only partially explained by the presence of lethal YY embryos since this class of embryos should comprise only 25% of the androgenetic embryo population. Preimplantation development was not adversely affected by technical manipulations in this experimental series since 45 of 48 (94%) control nuclear transplant embryos developed to the blastocyst stage.

The ability of androgenetic and gynogenetic and control blastocysts to undergo *in vitro* postimplantation development was assessed by transferring these embryos into Dulbecco's modified Eagle's medium. Androgenetic and gynogenetic embryos were observed to differ in two respects (Fig. 1). On day 2 of outgrowth 90% (28/31) of the gynogenetic, and all of the control (11/11) nuclear transplant blastocysts, had initiated blastocyst outgrowth. In contrast, only 10% (2/20) of day 2 androgenetic blastocysts had done so. Androgenetic embryo outgrowth was, however, observed on the following day. Thus, androgenetic embryos were observed to initiate blastocyst outgrowth approximately 1 day later than gynogenetic and control embryos. On day 6 of outgrowth, 26 of 31 gynogenetic embryos had degenerated and could no longer be observed and the remainder consisted of only a few cells. In contrast, 18 of the 20 androgenetic outgrowths and 10 of 11 control outgrowths remained intact on day 6. The androgenetic and control outgrowths were observed to undergo gradual degeneration during the subsequent 5 days of *in vitro* culture. Therefore, there appears to be a selective death of gynogenetic trophoblast cells *in vitro*, which parallels the paucity of extraembryonic tissue observed in gynogenetic embryos *in vivo* (Barton *et al.* 1984).

The demonstration that maternal and paternal genomes are programmed to function differently during development in mammals raises two possibilities. In one, maternal and paternal genomes may have been programmed to function differently during gametogenesis irrespective of each other or their shared cytoplasmic environment. Alternatively, appropriate gene action may depend upon intranuclear interactions so that activation of components of one parental genome is dependent upon the presence of the opposing parental genome. No evidence that would discriminate between these two possible mechanisms presently exists.

CONCLUSIONS

The mammalian embryo consists of haploid genomes inherited from the respective parents and membrane/cytoplasmic components inherited largely from the maternal parent. Successful development depends upon appropriate interaction of these three cellular compartments with each other and with their environment. The results of altering the nuclear/cytoplasmic components of the mouse embryo have revealed several important aspects of these reciprocal interactions that include: (a) male and female gamete nuclei are functionally distinct, (b) the newly formed embryonic nuclei interact with their cytoplasmic environment in a stochiometric and species-specific manner and (c) as development proceeds cleavage-stage mouse embryo nuclei rapidly lose their ability to support development when returned to zygote cytoplasm. Major goals of future investigations will be to determine how and precisely when the maternal and paternal gamete genomes are programmed to function differently and the mechanisms by which nuclear and cytoplasmic components mutually interact to result in appropriate gene action.

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