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Embryonic cytoplasmic extracts rescue murine androgenones to the blastocyst stage

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Summary

Androgenones (paternally derived genome) show a significant inability to form a blastocoele cavity. Eighty percent of these embryos die or arrest at earlier stages. Factor(s) from both normal and parthenogenetic late preimplantation embryos injected into each blastomere of androgenetic 4-cell stage can rescue more than twice as many to the blastocyst stage (47.2% versus 19.2% for non-injected androgenones). This factor(s) becomes available beginning at the 4-cell stage and is titratable. Injected total cytoplasmic mRNA will also cause a rescue response. Isolating this specific factor message(s) will permit the eventual cloning of possibly the earliest parentally imprinted gene(s) expressed during development.

Key words: androgenones, mouse preimplantation embryo, uniparental, blastocyst formation, cytoplasmic microinjections.

Introduction

Murine androgenones (paternally derived genome) and gynogenones (maternally derived genome) show extreme and lethal morphologies (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1986a). However, analysis of [^{35}S]methionine-labelled proteins separated on 1- and 2-dimensional electrophoresis gels show no consistent, discernible differences among androgenetic, gynogenetic and normal embryos (Petzoldt et al., 1981; Solter et al., 1986; Surani et al., 1986b; personal unpublished data). There are five possible reasons for this: (1) there are no de novo protein differences; (2) differences occur outside developmental stages analyzed; (3) the technique is not sensitive enough to detect the proteins in question; (4) the difference is not the lack of particular proteins, but inconsistent, relative reductions of these proteins, which are difficult to detect on gels of pooled embryos; or (5) the difference may be the lack of appropriate post-translational modifications, which are difficult to detect on nascent protein gels. We have been able to eliminate the first two possibilities by rescuing 4-cell androgenones to the blastocyst stage with cytoplasmic extracts. This is the first report that preimplantation androgenones differ fundamentally from normal and gynogenetic embryos at the molecular level.

Materials and methods

Production of androgenones

Androgenones were produced using techniques published previously (McGrath and Solter, 1983). Briefly, C57/BL6 × Balb/c F_1 (CBc F_1) murine zygotes were placed into M2 manipulation medium containing 7.5 μg/ml cytochalasin B (Sigma), 20 μg/ml demecholcine (Sigma) and 300 U/ml DNAase 1. After 5 minutes when the cytoskeleton had sufficiently depolymerized, the female pronucleus was removed from each zygote with a beveled and sharpened micropipet 20 μm in outer diameter. The female pronucleus was replaced with a male pronucleus and a small quantity of inactivated Sendai virus (American Type Tissue Collection) to facilitate fusion of the karyoplasts. Since prolonged exposure (2 hours) to demecholcine reduces development, the embryos were removed from the manipulation medium within 30-40 minutes. They were washed 1-2 minutes in fresh M2 and transferred to 100 μl drops of M16 for >30 minutes to remove all traces of the cytoskeletal inhibitors. Finally, the androgenones were placed into 50 μl drops of M16 (Day 1 of culture) and incubated at 37°C under a 5% CO_2 atmosphere for up to 5 days.

Preparation and injection of cytoplasmic extracts

Androgenones were cultured for 36 hours when ~75% had cleaved to 3- to 4-cell stage. Haploid C57/BL6 parthenogenones were activated (as described by Hillary et al., 1989) at 16.5 hours post HCG injection with 30 μM A23187 calcium ionophore for 5 minutes followed by 4 hours in 10 μg/ml cycloheximide. Zygotes showing single pronuclei were then washed and cultured for another 30 hours until they had reached the 8-cell stage. Both A23187 and cycloheximide have been used separately to parthenogenetically activate mammalian oocytes (Kaufman, 1978; Sirocusa et al., 1978). The combined use of these two reagents, however, significantly increases the rate of activation in a number of mouse strains

and results in haploid parthenogenones with one pronucleus (unpublished data).

Zona pellucidae of 40-50 in vitro cultured CBc F_1 2-cells (A/2C), 4- to 8-cells (A/4C), morula to blastocysts (A/50M) or 8-cell parthenogenones (A/G8C) were removed with acid Tyrode's (pH 2.5). The denuded embryos were then placed into 1 μl of double distilled H_2O (ddH_2O) on ice, allowing the osmotic shock to burst the cells. The crude extract was centrifuged at 4000 revs/minute for 10 minutes at 4°C to remove large debris. The supernatant was then loaded into microinjection pipets at 4°C and ~1 pl injected into the cytoplasm of each blastomere of 3- to 4-cell androgenones at room temperature. Each micropipet was removed from the cold for no longer than 15 minutes to minimize proteolysis. The injected androgenones were then returned to culture and their development to blastocyst (48 hours) observed. Considering that the normal morulae/blastocysts used to make the cytoplasmic extract contained an average of 32 cells each, then the dilution factor of any injected factor(s) can be estimated to be approximately 700-fold ([1 μl/1 pl/(32 cells × 50 embryos)/blastomere]).

Messenger RNA was isolated from 150-180 morula/blastocysts using a Micro Fast Track mRNA purification kit (Invitrogen) and resolublized in 1.5 μl ddH_2O. The solution was loaded into microinjection pipets and injected into androgenones as described above.

Controls consisted of non-injected normal (N) and androgenetic embryos (A), normal and androgenetic 4-cells injected with phosphate-buffered saline (pH 7.4, ~300 mosm; N/PBS; A/PBS) and normal 4-cells injected with morula extract supernatant (N/50M).

Androgenones were observed by light microscopy and scored for stage-specific morphologies. Regardless of cell number, those embryos cavitating and/or compacting were called blastocysts (B) and morulae (M), respectively. 3- to 4-cells (4C) and 8-cells (8C) showed defined blastomeres. Dead embryos (D) no longer contained an intact plasma membrane (Fig. 1). Use of these standard terms does not suggest that we consider these embryos normal, but that they have exhibited the gross observational criteria for the development stage to which they were assigned. However, injection of cellular extracts does restore some aspects of normal early development. As will be discussed in a subsequent paper (Hagemann, Navara and First, unpublished data), we found that the mean cell number of injected androgenome blastocysts approached that of normal blastocysts, and was significantly higher than that of non-injected androgenone blastocysts. Also certain developmental events such as blastomere polarization, absent in non-injected androgenones, was observed in extract-injected ones.

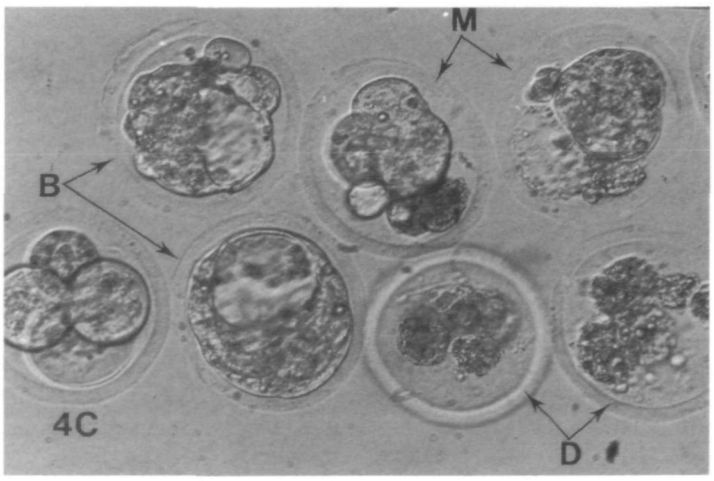

Fig. 1. Development in culture of androgenones injected with embryonic cytoplasmic extracts. B, blastocyst; M, morula; 4C, 4-cell stage; D, dead.

Data analysis

Data from 3-6 replicates of 20-40 embryos each were pooled and an overall average percent blastocyst formation shown. Data were normalized by the arc-sin transformation and analyzed by the GLM (General Linear Models) procedure of SAS (1985). Means were separated by Duncan's multiple range test.

Results

Androgenones were placed into culture and their development to blastocysts observed. Fig. 2 shows that on day 3 of culture (D3), approximately 25% of the androgenones had blocked at the 2-cell stage probably due to random, lethal combinations of two Y-bearing pronuclei. These arrested embryos were removed and the remaining androgenones observed further. On day 4, those androgenones that had cleaved to 3- to 4-cells on the previous day began to show delayed development (only 63.5% morula) and death (0.9%). By day 5, the death rate increased (to 36.5%) as the developmental potential decreased (19.2% blastocysts). There appeared to be parentally imprinted proteins necessary as early as the 8-cell to morula stages that were not being transcribed by the paternal genome.

To test this hypothesis, a crude extract of water-soluble cytoplasmic components was made from normal morula/early blastocyst embryos and injected into each blastomere of 3- to 4-cell androgenones on day 3 of culture (Fig. 3). After 48 hours incubation, more than twice as many androgenetic embryos showed a normal progression to the blastocyst stage (47.2% versus 19.2% and 20.5% for non-injected and PBS-injected andro-

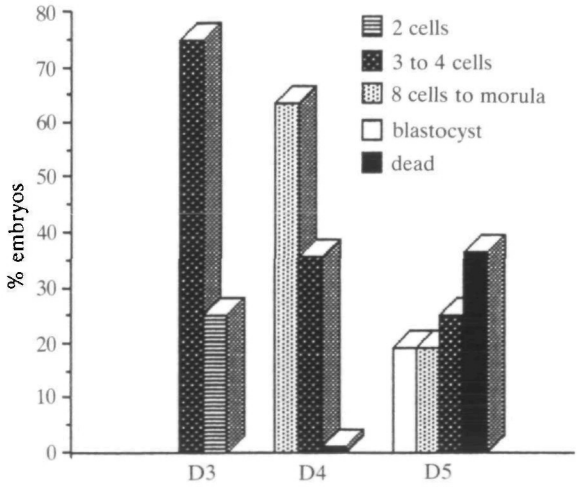

Fig. 2. Development of androgenones in culture. D3, D4 and D5, day 3, 4 and 5 in culture, respectively. Embryos arresting at 2-cell stage on D3 were removed and data from D4 and D5 normalized to the remaining 3 to 4 cells. $n=104$ embryos over 6 replicates.

genetic controls, respectively; Table 1). The factor(s) in the extract that rescued these androgenones appeared to become available beginning at the 4- to 8-cell stages (31.6% blastocysts). It was also found present in parthenogenetic 8-cells in the same quantity as that of normal embryos of a similar developmental stage (47.5% blastocysts). A partial purification of the normal morula/blastocyst extract into its mRNA component resulted in a significantly increased response when injected into androgenone blastomeres compared to non-injected androgenone controls (33.3% blastocysts).

For all extracts showing a significant improvement in development, there was also an accompanying drop in embryonic death (Fig. 4; 36.5% versus 24.5% for non-injected and morula-injected androgenones, respectively). The decrease in mortality, however, does not account for all of the increase in blastocyst formation. The remaining portion results from the slight decline in the number of embryos arrested at the morula, 8-cell or 3- to 4-cell stages.

To titrate the suspected factor(s), extracts were made and injected as described above, except that 25

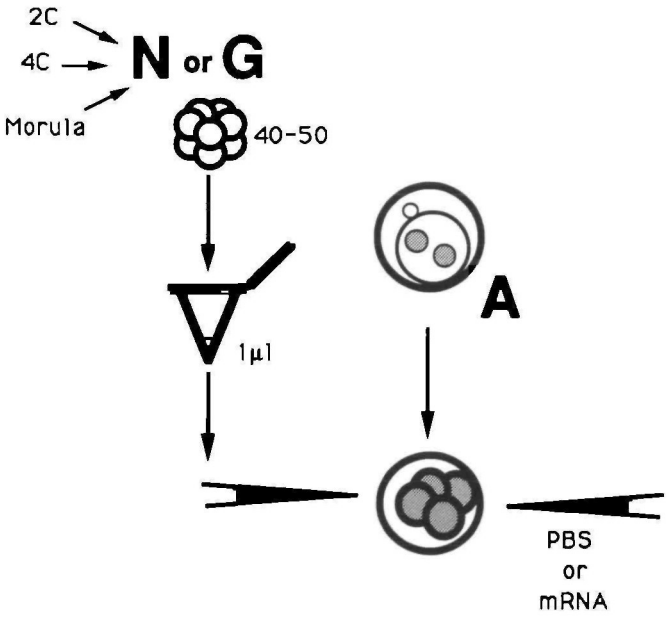

Fig. 3. Preparation of crude cytoplasmic extracts. Gynogenetic and normal 2-cell stage (2C), 4-cell stage (4C), and morula embryos were denuded and placed into 1 ml dd H_2O. After centrifugation, the extract was injected into each blastomere of 4-cell androgenones. Alternatively, phosphate-buffered saline (PBS) or purified total cytoplasmic mRNA was injected.

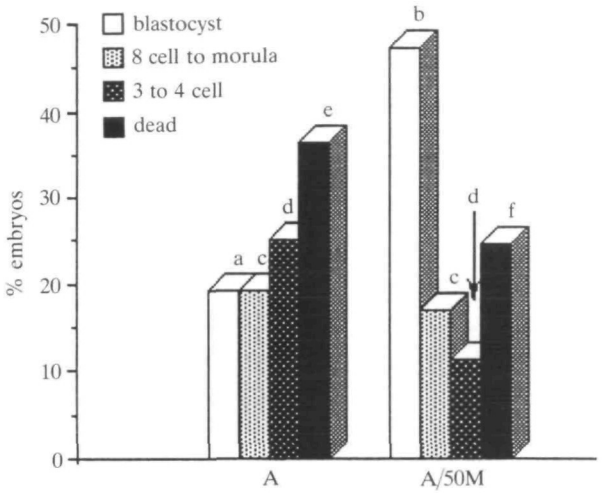

Fig. 4. Comparison of developmental potential between injected and non-injected androgenones. A, non-injected androgenones (n=104); A/50M, androgenones injected with morula extract (n=104). Data analyzed within developmental stages. a and b, c, d, e and f, treatments showing significant differences within stages (P<0.01).

Table 1. *Development of normal and adrogenetic 4-cell embryos injected with cellular extracts*

	Development								
		day 4			day 5				
Treatment	day 3 3-4C*†	8C-M	3-4C	D	B	8C-M	3-4C	D	%B‡
N	144	143	1	0	138	6	0	0	95.8[a]
N/PBS	143	143	0	0	138	5	0	0	96.5[a]
N/50M	123	119	0	4	116	3	0	4	94.3[a]
A	104	66	37	1	20	20	26	38	19.2[b]
A/PBS	88	57	30	1	18	14	4	52	20.5[b]
A/2C	76	48	25	3	14	24	14	24	18.4[b]
A/4-8C	98	63	26	9	31	25	14	28	31.6[c]
A/G8C	61	44	12	5	29	6	6	20	47.5[d]
A/50M	106	82	22	2	50	18	12	26	47.2[d]
A/mRNA	63	31	31	1	21	17	15	10	33.3[c]

*3-4C, 3- to 4-cell stage; 8C-M, 8-cell to morula stages; D, dead; B, blastocyst stage; N, non-injected normal embryos; N/PBS, normal embryos injected with phosphate-buffered saline; N/50M, normal embryos injected with morula extract; A, non-injected androgenones; A/PBS, androgenones injected with PBS; A/2C, androgenones injected with 2-cell extract; A/4-8C, androgenones injected with 4- to 8-cell extract; A/G8C, androgenones injected with parthenogenetic 8-cell extract; A/50M, androgenones injected with morula extract; A/mRNA, androgenones injected with purified morula mRNA.

†indicates total number of embryos injected and observed for each treatment (n).

‡indicates per cent of injected embryos (3-4C on day 3) developing to blastocyst stage on day 5 ([B/n]×100).

[a,b,c,d]treatments showing significant differences, P<0.01.

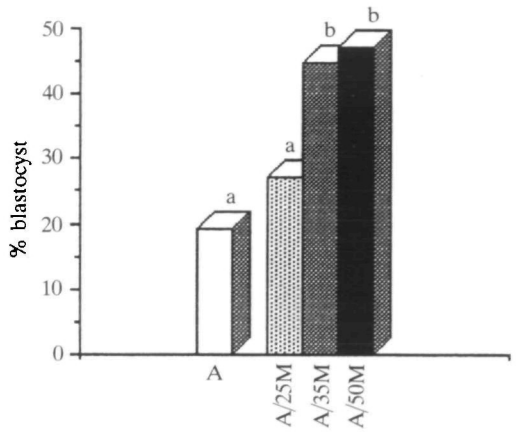

Fig. 5. Titration of cytoplasmic extracts. A, non-injected androgenones; 25M, 25 morula per 1 μl ddH_2O; 35M, 35 morula per 1 μl ddH_2O; 50M, 50 morula per 1 μl ddH_2O. n=104 (A), 103 (A/25M), 96 (A/35M) and 106 (A/50M), respectively, a,b, treatments showing significant difference (P<0.01).

(A/25M) or 35 (A/35M) denuded morulae were added to 1 μl ddH_2O instead of the standard 50. Fig. 5 shows that as the number of morula/blastocysts increased, there was also an increased response (27.2% and 44.8% blastocyst, respectively) in a step-wise fashion.

Discussion

Although androgenones can develop to implantation with trophoblast hypertrophy and little to no fetal development (Barton et al., 1984), this is the extreme limit of their developmental capabilities. In reality, only approximately 20% of those dividing to 4-cell stage will continue to form blastocysts, compared to 80-90% for gynogenetic and normal embryos, respectively. By injecting embryonic cytoplasmic component(s) into blastomeres of androgenetic 4-cells, we have been able to rescue androgenones from developmental arrest and death. This component(s) or factor(s) appears to be expressed beginning at 4 cells and increases in concentration during development through at least the morula stage. This factor(s) is likely an exclusively paternally imprinted gene(s) since parthenogenones can rescue these androgenones at the same rate as normal embryos.

The macromolecular nature of this factor(s) is not completely known. However, the developmental pattern observed when total cytoplasmic mRNA was injected into the androgenetic blastomeres, was very similar to that seen for the injected 4-cell extract. This suggests that the gene(s) is transcribed beginning at the 4-cell stage and its message(s) later translated during the 8-cell to morula stages. The availability of this message(s) will facilitate the eventual cloning of this early preimplantation parentally imprinted gene(s).

The range of stages at which androgenones arrest development and die suggests that the imprinting modification is leaky. A low level of necessary protein(s) is transcribed in the developing androgenone. A certain number of these embryos (~20%) acquire threshold quantities of this protein(s) to develop to the blastocyst stage; 80% do not. Titration of the morula extract also lends support for this hypothesis. Originally, we intended to retain the same number of embryos used in each extract by replacing a portion of the standard 50 morulae with 2-cells, as the latter stage did not cause a response. However, when extracts of 25 morulae/blastocysts plus 25 2-cell stages (50%) or 35 morulae/blastocysts plus 10 2-cell stages (75%) were injected, both experiments resulted in developmental potentials no different than that of non-injected androgenones (data not shown). Extracts were then made by simply reducing the number of morulae/blastocysts to 25 or 35 without the addition of 2-cells. It is likely that very early preimplantation stages cause inhibition of the injected factor(s). Consequently, the factor's(s') nascent availability at the 4-cell stage may result from either the initiation of its transcription and subsequent translation, or a decline in its inhibition. McGrath and Solter (1986) and Howlett et al. (1987) observed that removal of a portion of the cytoplasm from haploid androgenones and gynogenones increased the embryos' rates of development. It is unclear, however, how this procedure can improve development. One possibility may be that removing cytoplasm also removes inhibiting factor(s) that would otherwise prevent development past the 2-cell stage. Restoration of this early cytoplasm should re-establish inhibition. Although we injected 2-cell stage cytoplasmic extracts, we found neither an increase nor decrease in development in our diploid androgenones. Likewise, we did not permanently change the blastomere volume as was done in these previous studies. These observations of others may, instead, result from alteration of the nuclear/cytoplasmic volume ratio. Consequently, we are cautious about suggesting a direct relationship between these previous results and our present study.

This rescuing factor(s) that is transcribed and translated during preimplantation from the maternal genome, may be the earliest expressed parentally imprinted gene(s) during murine embryonic development. Thus far, there are 3 genes known to be parentally imprinted in the mouse. All of them are expressed peri- or post-implantation. These include the *H19* gene (Bartolomei et al., 1991), as well as the insulin-like growth factor II (DeChiara et al., 1990) and its receptor (Barlow et al., 1991).

The eventual cloning and study of the rescuing gene(s) presented here may help in elucidating the imprinting mechanism. The incomplete or leaky nature of this imprinting modification may imply that parental imprinting is an important contributor towards individual variation. Diversity among individuals within a species is paramount in evolution and sexual reproduction. Imprinting may be a subtle, but powerful method for parents to improve cooperatively the survivability of their offspring.

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