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A Y-chromosomal effect on blastocyst cell number in mice

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SUMMARY

Karyotypic and cell number analysis of 3.5 day post coitum preimplantation mouse embryos was used to determine whether XY embryos had more cells than XX embryos at the late morula/early blastocyst stage. This proved to be the case for the CD1 strain (for which it had previously been shown that XY embryos form a blastocoel earlier than XX embryos) and for the MF1 strain. However, this increased cell number was not seen in MF1 embryos carrying an RIII strain Y in place of the MF1 Y. Furthermore, interstrain crosses between CD1 and the MF1,Y^{RIII} strain showed that the cell number increase segregated with the CD1 Y but not with the RIII Y. It is concluded that the CD1 and MF1 Y chromosomes carry a factor that accelerates the rate of preimplantation development.

Key words: preimplantation embryos, XX-XY differences, preimplantation Y chromosome effect

INTRODUCTION

It has become apparent from a number of lines of evidence that, in mammals, there are some phenotypic differences between the sexes before the development of the gonads, which cannot therefore be a consequence of gonadal hormone action. In marsupials, sexual dimorphisms relating to the development of scrotum and mammary glands appear prior to gonadal sex differentiation and have been shown to be hormonally independent (Renfree and Short, 1988; Shaw et al., 1990). In eutherian mammals, the pregonadal sexual dimorphism is more subtle and concerns a difference in developmental rate, XY individuals being developmentally more advanced than XX individuals at the same gestational age. This developmental difference has been reported as a weight difference in 12.5 day post coitum (dpc) rats (Scott and Holson, 1977), as a difference in the number of somites in 8.5 dpc mice (Seller and Perkins-Cole, 1987) and as a difference in body length and head size in first trimester human fetuses (Pedersen, 1980). Preimplantation XY embryos have also been reported to be more advanced than XX embryos in mice (Tsunoda et al., 1985; Gardner and Leese, 1987) and in cattle (Avery et al., 1991; Xu et al., 1992).

In principle, these pregonadal XX-XY differences could be due to the difference in X chromosome constitution and/or to an effect of the Y chromosome. In a study presented elsewhere (Thornhill and Burgoyne, 1993), we have shown that a paternally derived X chromosome has a retarding effect on development in mice. We believe that this provides an explanation for the developmental retardation of XX embryos relative to XY embryos as manifested in the postimplantation period (prior to gonadal differentiation). In contrast, the results reported here show that the preimplantation XX-XY difference reported by Tsunoda et al. (1985) is due to an accelerating effect of the CD1 Y chromosome.

MATERIALS AND METHODS

Mice

The crosses used to provide embryos were as follows.

(1) MF1 (random bred albino from OLAC) females mated to MF1,Y-del^{RIII} males. The Y deletion was first identified in an Sxr^a carrier male and was backcrossed for more than 10 generations to a random-bred MF1 background. A normal Y from a brother was also backcrossed to MF1 and this is utilised in cross 2. The Y chromosome in the Sxr^a stock is known to have originated from the RIII inbred strain (Tease and Cattanach, 1989). The Y-del^{RIII} chromosome has a large deletion from the long arm, which has removed over half the copies of the Y-specific long arm sequence Y353B (Bishop et al., 1985; Burgoyne et al., unpublished). This type of male was initially chosen because the small size of the Y-del^{RIII} chromosome facilitates recognition in metaphase spreads, and in the knowledge that a clearcut XX-XY difference was present at 10.5 dpc with this cross (Burgoyne, unpublished).

(2) MF1 females mated to MF1,Y^{RIII} males.

(3) CD1 (random-bred albino from Charles River) females mated to CD1 males.

(4) MF1 females mated to CD1 males.

(5) MF1 females mated to (MF1 × CD1)F_1 males.

(6) CD1 females mated to (CD1 × MF1,Y-del^{RIII})F_1 males.

(7) MF1 females mated to MF1 males.

The females were killed in the morning or early afternoon of the fourth day of pregnancy (day of plug is the first day), and the embryos were flushed from the female tract with M2 (Whittingham, 1971) medium. One female from cross 1 was killed at 10

am on the third day of pregnancy, the embryos were flushed from the oviduct in M2 and placed individually in drops of M16 (Whittingham, 1971) medium under paraffin oil. These embryos were cultured at 37°C in 5% CO_2 in air and were scored at intervals for the presence of a blastocoel. All embryos were processed as described below.

Preimplantation embryo preparations

The embryos were placed individually in drops of M16 containing approximately 0.08 μg/ml of colcemid (Sigma) and incubated under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. In the early part of the study, embryos were processed 1-2 hours later; however, a number of embryos lacked divisions. Leaving all the embryos longer resulted in some embryos having so many divisions that they tended to merge, making cell and chromosome counts difficult. These difficulties were largely overcome by examining the embryos at intervals of 1 hour with an Olympus CK inverted microscope (the stage was kept at approx. 37°C with a heating pad) and processing the embryos when they had developed rounded blastomeres at the surface (indicative of dividing cells).

To process, an embryo was washed in a drop of M2 medium and placed in a drop of 1% trisodium citrate for 2.5 minutes. The embryo was then transferred in a fine glass pipette containing 0.75% trisodium citrate to a clean glass slide. While observing under a Wild M5 dissection microscope, two drops of 'aqueous fixative' (9:3:2 methanol:glacial acetic acid:distilled water) were added before the citrate drop had time to dry. The aqueous fixative dissolves the zona, and when the embryo started to 'collapse' one drop of 3:1 methanol:glacial acetic acid fixative was added to drive away the aqueous fix. (Technical note: for embryos with less than 16 cells only a single drop of aqueous fix is needed. For late blastocyts, three drops of aqueous fix and two drops of 3:1 fix are required. If the latter two drops are each placed a short distance from, rather than on the embryo, and on opposing sides, the 'washing' action of the fix helps to clear away the cytoplasm.) The preparation was then blown dry and stained in 2% Giemsa in pH 6.8 phosphate buffer. Cell counts were carried out under a ×16 objective, and metaphases were analysed under a ×100 oil immersion objective. Some slides were destained and C-banded (Evans, 1987) to check the initial diagnosis.

RESULTS

Of 548 embryos from the fourth day of pregnancy, 15 were excluded because of gross abnormalities (usually dead or fragmenting blastomeres), 10 were lost during processing and 62 lacked metaphases. The majority (50) of those lacking metaphases had cell numbers (1×8-cell, 5×16-cell, 37×32-cell, 7×64-cell) which equate with successive doublings, suggesting that there is still considerable synchrony of cell division up to the 64-cell stage. Of the 447 normal embryos with metaphases, all provided cell counts and 402 (90%) provided chromosome counts. Of these 402, 22 were aneuploid and have been excluded from the XX v XY comparisons. These aneuploid embryos were in nearly all cases retarded compared to litter mates, which raises the possibility that the retarded 8- and 16-cell embryos lacking metaphases were also aneuploid. Scoring for the presence of a Y chromosome was a simple matter with the marker Y-del chromosome. It is less easy with a normal Y (Fig. 1); nevertheless, of 263 embryos scored, a confident or reasonably confident diagnosis was made in 218 (83%). The main factor affecting confidence was the number of metaphases available. 37 preparations were destained and C-banded because of a lack of confidence in the original scoring, and in 34 the C-banding confirmed the original tentative diagnosis.

Because cell numbers are increasing exponentially, the data have been log-transformed for analysis. The XX v XY comparisons were made using mean weighted differences calculated from within litters as previously described (Burgoyne et al., 1983) and the results for all the crosses are presented in Table 1. The initial result obtained with the MF1 × MF1,Y-del^{RIII} cross was surprising because it indicated no difference in cell number between XX and XY-del embryos at the morula/blastocyst stage (Fig. 2A), although this strain shows a clear XX v XY-del difference at 10.5 dpc. Since the XX v XY difference in the CD1 strain (Tsunoda et al., 1985) was observed with respect to the timing of blastocoel formation rather than cell number, one litter of embryos from the MF1 × MF1,Y-del cross was cultured from the 4-cell stage and scored for blastocoel formation before being karyotyped. As can be seen from Table 2, there is an almost perfect correlation between the order in which embryos form a blastocoel and the ranking of the embryos in terms of cell number when processed. Nevertheless, there is once again no correlation with genotype. Since the Y-del chromosome is deleted for much of

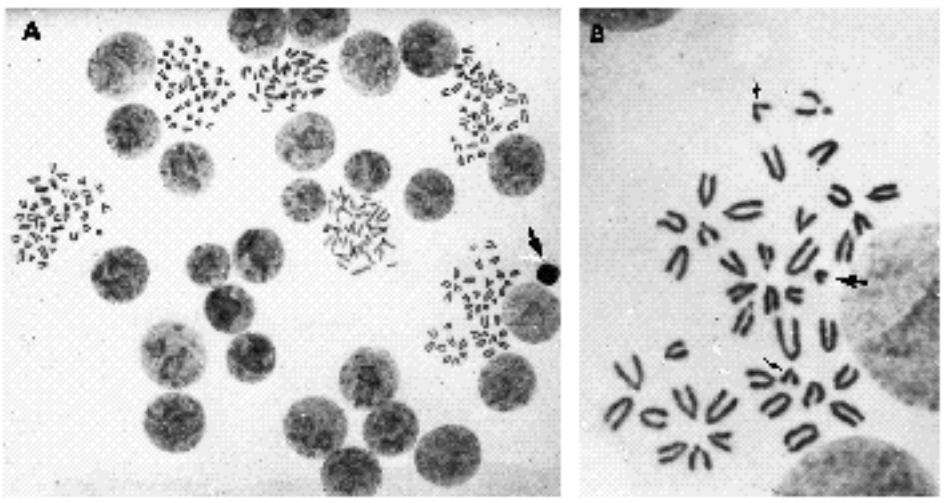

Fig. 1. An air-dried preparation of a 32-cell morula from the CD1 strain. (A) Low-power view of the whole embryo showing 26 interphase and 6 metaphase nuclei. A pycnotic polar body nucleus is also present (arrow). (B) A high power view of a metaphase in which the two chromosome 19s (small arrows) and the Y chromosome (large arrow) are clearly visible. The Y could be identified in 5 of the 6 metaphases present.

Table 1. Mean log cell numbers and estimated XX-XY differences for 3.5 dpc XX and XY embryos from seven different crosses

Cross	Number of embryos XY	Number of embryos XX	$\bar{X}$ ± s.e.m.* log cell number XY	$\bar{X}$ ± s.e.m.* log cell number XX	$\bar{X}$ ± s.e.m.† × 10^3 weighted difference	Sign. (*P*)‡ of difference
MF1 × MF1,Y-del^{RIII}	31	44	1.58 ± 0.04	1.60 ± 0.04	−12 ± 20	NS
MF1 × MF1,Y^{RIII}	24	18	1.60 ± 0.03	1.60 ± 0.03	9 ± 22	NS
CD1 × CD1	19	13	1.65 ± 0.05	1.57 ± 0.06	74 ± 22	<0.005
MF1 × CD1	10	17	1.73 ± 0.02	1.61 ± 0.02	119 ± 37	<0.005
MF1 × (MF1 × CD1)F_1	35	26	1.69 ± 0.03	1.63 ± 0.03	63 ± 20	<0.005
CD1 × (CD1 × MF1,Y-del^{RIII})F_1	20	36	1.64 ± 0.05	1.65 ± 0.04	−5 ± 24	NS
MF1 × MF1	21	26	1.67 ± 0.03	1.59 ± 0.04	71 ± 21	<0.005

*Mean of litter means.
†The error for the mean weighted difference is calculated using the variance within genotypes within litters.
‡Significance by Student's *t*-test.

Table 2. Time of blastocoel formation in relation to karyotype and cell number when processed for one litter of MF1 × MF1,Y-del^{RIII} embryos

Time blastocoel first observed*	Number of embryos	Karyotype	Cell no. when processed†
17.00h 4th day	1	XX	73
18.00h 4th day	1	XX	71
19.00h 4th day	1	XY	61
20.30h 4th day	1	XX	42
21.00h 4th day	2	XX,XY	41,30
8.45h 5th day	4	XY,?,XY,XY	46,31,28,25

*Embryos were scored half hourly until 21.30 hours on day 4, and then at 8.45 hours the following morning.
†All embryos were placed in colcemid-containing medium at 8.45 hours on the 4th day. The cell number when processed therefore equates with the number of cells when the colcemid was added.

the long arm of the Y, it was possible that the deletion had removed information on the Y needed for the accelerated preimplantation development. However, the results from the MF1 × MF1,Y^{RIII} cross (Fig. 2B) also show no XX v XY difference.

In the light of these initial results, some CD1 mice were obtained, and the results for this strain confirm the finding of Tsunoda et al. (1985) that XY embryos are more advanced than XX embryos (Fig. 2D), the difference being highly significant (*P*=0.005-0.001).

This clearcut difference between the MF1,Y^{RIII} and CD1 crosses can be used to test whether it is the CD1 Y which is responsible for the accelerated development. From Fig. 2C,E,F and Table 1, it is clear that the XX v XY difference is linked to the CD1 Y. The most important comparison is between the results for cross 5 [MF1 × (MF1×CD1)F_1] where all the X chromosomes are MF1 but the Y is CD1, and cross 6 [CD1 × (CD1×MF1,Y-del^{RIII})F_1] where CD1 X chromosomes are combined with the Y-del^{RIII}. For cross 5, there is a clear XX-XY difference (*P*=0.005-0.001) while in cross 6 there is no XX-XY difference.

The CD1 Y is of the *domesticus* type (Gubbay et al., 1990) whereas the RIII Y is of the *musculus* type (Mardon et al., 1989), raising the possibility that we are dealing here with a *domesticus* v *musculus* difference. However, the results in Fig. 2G for the cross involving normal MF1 strain

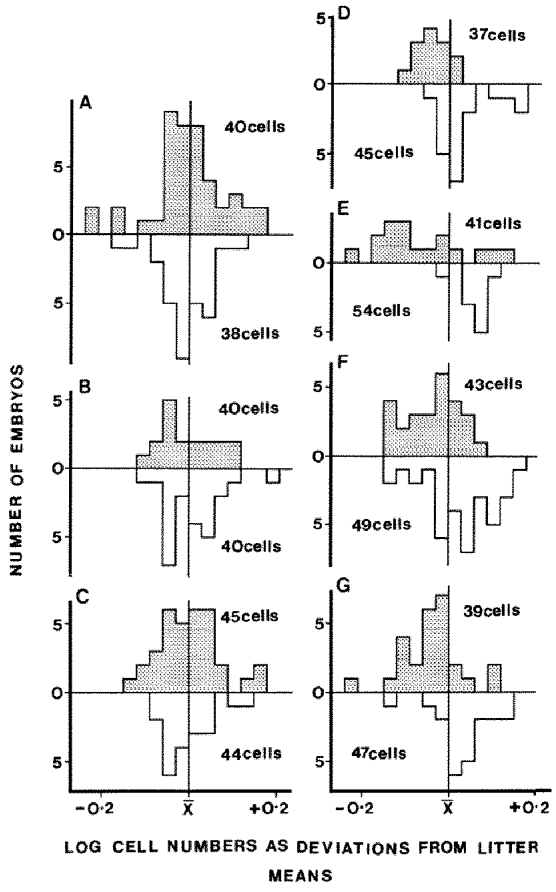

Fig. 2. The log cell number data for 3.5 dpc preimplantation embryos from 7 different crosses plotted as deviations from 'litter means' [$\bar{X}=(\bar{X}_{xx}+\bar{X}_{xy})\div 2$]. For each cross, the XX data are plotted above the line and the XY data below the line, and the estimated mean XX and XY cell numbers are given (i.e. the antilogs of the means of litter means from Table 2). The crosses showing no significant XX v XY difference are plotted on the left, and those with a significant difference on the right. (A) MF1 × MF1,Y-del^{RIII}. (B) MF1 × MF1,Y^{RIII}. (C) CD1 × (CD1 × MF1,Y-del^{RIII})F_1. (D) CD1 × CD1. (E) MF1 × CD1. (F) MF1 × (MF1×CD1)F_1. (G) MF1 × MF1.

males show a clearcut XX v XY difference even though the Y involved is of the *musculus*-type. [The two stud males used were kindly typed by Dr Susan Darling, and shown to have the *musculus*-type *Zfy* pattern (Gubbay et al., 1990) in a Southern blot of *Taq*I restricted DNA.]

DISCUSSION

The present results demonstrate that the preimplantation XX-XY difference in the CD1 strain, originally reported by Tsunoda et al. (1985), is due to an effect of the CD1 Y chromosome. This XX-XY difference is also seen with an MF1 Y chromosome, but is not seen with an RIII Y chromosome, at least in the context of an MF1 or mixed MF1/CD1 strain background.

How might the Y chromosome be exerting this effect on preimplantation development? Three possibilities are discussed here; namely (a) earlier fertilisation by Y-bearing than X-bearing sperm, (b) more rapid completion of DNA replication in XY cells because of the reduced DNA content of the Y as compared to the X, (c) expression of a Y gene(s) that speeds up the rate of cell division.

The first possibility is undermined by data that we have obtained at the 2- to 4-cell division in a cross involving females heterozygous for the large X inversion In(X)1H and MF1 males (P. S. Burgoyne and E. P. Evans, unpublished). These females produce a high frequency of nullo-X eggs and the purpose of the study was to examine the early development of XO and OY embryos. In the present context, it is the order of entry of the XX and XY embryos into the second cleavage division that is of relevance. Splitting the embryos into early, intermediate and late cleaving groups to allow comparison with the cavitation data of Tsunoda et al. (1985), the percentage of XY embryos was 59% (n=32), 51% (35) and 50% (28) respectively. This compares with the 71%, 44% and 20% males from Tsunoda's data on the timing of blastocoel cavity formation. This suggests that the XX-XY difference is not present at fertilisation, but develops as cleavage progresses. This is supported by the findings of Avery et al. (1991) and Xu et al. (1992) that among bovine blastocysts produced by in vitro fertilisation the XY blastocysts have more cells and are more advanced than XX blastocysts, even though the X- and Y-bearing sperm will have come in contact with the eggs at the same time.

The second possibility is also unlikely in view of our finding that the RIII Y, even when much of the long arm had been deleted, was not associated with an increase in cell number. It therefore seems likely that we are dealing with a positive effect of a Y gene on the rate of cell division.

It is known that serologically detected male antigen (SDMA, which may or may not be the same as H-Y) is expressed in male preimplantation embryos (Epstein et al., 1980; White et al., 1982; Shelton and Goldberg, 1984) and that the expression of this antigen requires a gene that is present in Sxr^a (Goldberg et al., 1991) and which must therefore be located on the short arm of the mouse Y chromosome (McLaren et al., 1988; Roberts et al., 1988). *Zfy-1* may also be expressed during preimplantation development since it has been shown to be expressed in embryo-derived stem cells (Koopman et al., 1989). Not enough is known about either of these genes to be able to judge whether they might influence the rate of cell division. A mouse Y-chromosomal gene encoding a ubiquitin-activating enzyme (now designated as *Ube1y-1*), which is thought to affect cell division, has recently been cloned (Kay et al., 1991; Mitchell et al., 1991), but it remains to be seen whether it is expressed in the preimplantation period.

The RIII Y chromosome involved in the crosses described here originated from XY males in our laboratory stock segregating for Sxr^a. Why should this Y produce no XX-XY preimplantation difference? This strain of Y has been shown to have functional copies of all three of the Y-chromosomal genes considered above, and we know these genes are still present on the RIII Y of our stock. It therefore seems likely that we are dealing with allelic differences between genes on the RIII Y as compared to the CD1 and MF1 Y chromosomes. Because the CD1 and RIII Y chromosomes are from different subspecies, it is expected (and indeed observed) that they will differ considerably at the DNA sequence level. However, a comparison of the more closely related MF1 and RIII Y chromosomes with respect to the DNA sequences for genes such as *Zfy-1* and *Ube1y-1*, might provide some clues as to which Y gene is responsible for thc preimplantation Y effect.

It is worth considering whether this preimplantation Y-chromosome effect is serving any definable male-specific function. Mittwoch (1969; 1989) has argued that the role of the Y in testis determination is mediated through a growth promoting effect of the Y, and has used the evidence of XX-XY differences that precede gonadal differentiation to support her thesis. However, we have shown that the size advantage of XY embryos seen at 10.5 dpc is neither necessary nor sufficient for testis determination. Thus 10.5 dpc *Sry*-negative XY fetuses (i.e. lacking the testis determinant - Gubbay et al., 1990; Koopman et al., 1991) are larger than their XX sibs but nevertheless develop as females, while 10.5 dpc *Sry*-positive XXSxr fetuses are the same size as their XX sibs but nevertheless develop as males (P.S. Burgoyne et al. unpublished). In many mammalian species, males are considerably bigger than females, and this is presumed to be related to their reproductive fitness. There may therefore have been selection in favour of factors that increase male size with respect to female size. However, in the example described here, and in the case of the XX-XY difference generated through the X-imprinting effect described by Thornhill and Burgoyne (1993), the XY size advantage is correlated with a difference in developmental stage - the XY embryos/fetuses are not large for their stage - so it is a moot point whether the early size advantage is translated into an increase in adult size.

One possibility suggested by Dr A. McLaren (personal communication), is that the early developmental advantage of XY over XX embryos may weight things in favour of XY embryos at the time of implantation, so that more XY than XX embryos implant. This would serve to compensate for a subsequent higher rate of loss of XY as compared to XX fetuses, as is known to occur in man.

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