

## Effect of gonadotropins on chromosome aneuploidy, chromosome mosaicism & sex ratio in mouse preimplantation embryos

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**Background & objectives:** There are potential risks of major birth defect in IVF (*in vitro* fertilization) pregnancy as well as IVF-ICSI (intra cytoplasmic sperm injection) pregnancies in comparison with naturally conceived human pregnancies. This increase risk could be due to either gonadotropins used for ovarian stimulation or *in vitro* culture conditions or multiple pregnancy or combinations of all the factors. The effects of gonadotropins on chromosome aneuploidy, chromosome mosaicism and sex ratio on mouse preimplantation embryos were evaluated through the use of fluorescence *in situ* hybridization (FISH).

**Methods:** The study material consisted of 111 preimplantation mouse embryos (2-16 cell stage) in control group and 405 preimplantation mouse embryos in gonadotropin stimulated group from genetically identical Swiss Albino young (6-8 wk) mouse kept in a similar environmental conditions. The study was designed to investigate effect of gonadotropins on chromosome aneuploidy, chromosome mosaicism and sex ratio through the use of FISH technique using chromosome X, Y and 19 probes. All blastomeres of embryos in both groups were assessed.

**Results:** Interpretable FISH results were obtained in 66 embryos in control group and 128 embryos in gonadotropin stimulated group. There was no excess of chromosome aneuploidy (only one case of sex chromosome trisomy in study group; 19, 19, X, Y, Y) or chromosome mosaicism or deviations in sex ratio between the two groups. However, deviation (1.36 M: 1 F in control group & 1.25 M: 1 F in study group) was seen from expected sex ratio (1 M: 1 F) *i.e.*, skewed sex ratio in both the groups.

**Interpretation & conclusions:** Our results showed that gonadotropins used for ovarian stimulation had no effects in causing increase in chromosome X, Y, 19 aneuploidy and mosaicism and skewing of sex ratio in mouse model. A large scale study with more FISH probes on a larger sample size need to be done to confirm the findings.

**Key words** Chromosome aneuploidy - fluorescence *in situ* hybridization - mosaicism - mouse preimplantation embryo - sex ratio

The majority of early pregnancy wastages are caused by chromosome abnormality<sup>1</sup>. Studies on human embryos collected from *in vitro* fertilization (IVF) cycles confirm high incidence of developmental arrest, embryonic aneuploidy and mosaicism<sup>2-4</sup>. Exogenous administration

of gonadotropins used for ovarian stimulation results in higher concentration of circulating steroids, which may affect oocyte and/or embryo<sup>5</sup>. This is supported by the study using milder ovarian stimulation for IVF that reduces aneuploidy rate in the human preimplantation embryo<sup>6</sup>.

However, all these studies were based on embryos from infertile patients undergoing superovulation and IVF. No information is available for preimplantation embryo derived from natural conception for comparison. It is difficult (due to technical as well as ethical reasons) to obtain preimplantation embryos derived from natural conception in human. Similarly it is difficult to obtain genetically identical inbred population and identical environmental condition in human. Hence, there is a need for research in a system that can eliminate these factors. Mouse model can overcome several factors like advanced maternal age, genetic heterogeneity, *in vitro* mechanized environment, infertility, dietary variations, ethnic differences, environmental differences and male factors through selecting fertile inbred of young (6-8 wk) healthy mice, giving same diet and keeping in the same environmental condition.

Although in general it is not possible to extrapolate from animal models to human, but considerable similarities exist among many mammalian species in early stages of development with respect to morphology and metabolism<sup>7</sup>. This suggests that information obtained from preimplantation embryos of laboratory animals might be applicable to those of the human<sup>7</sup>.

In this study we therefore, have chosen a mouse model to investigate gonadotropin effect on chromosome aneuploidy (deviation from normal number), chromosome mosaicism (two or more cell lines derived from a single zygote) and sex ratio (number of males to that of females) in early preimplantation embryo using fluorescence *in situ* hybridization (FISH).

### Material & Methods

This study was carried out in the Department of Reproductive Biology, All India Institute of Medical Sciences (AIIMS), New Delhi, from September 2003 to June 2007. Swiss albino mice of 6-8 wk old (inbred and maintained in captivity for several generations) were obtained from animal house of AIIMS and used for the study. The study was approved by the animal ethics committee of AIIMS.

Commercially available preparations of gonadotropins were used for inducing superovulation. Gonadotropins used in the study for follicle stimulation were pregnant mare serum gonadotropin (PMSG; Sigma, India), folligon (contains both FSH & LH;

Intervet, Netherlands), pergonal (human menopausal gonadotropin; Serono, USA) and for induction of ovulation was human chorionic gonadotropin (hCG; Sigma, India).

Superovulation was induced by injecting intraperitoneal (ip) 5, 10 or 15 IU of folligon or other gonadotropins whereas induction of ovulation was carried out (after 48 h) by injecting 5 IU of hCG. Different commercially available ovulation inducing agents were used in the study to test whether there is any difference in causing chromosome anomalies due to their different mode of preparation (urine, serum, etc). Different dosages of the same drug were used to find out dose related effect. In order to obtain embryos from natural cycle females mice of 6-8 wk age were not injected with any gonadotropins and oestrous were identified by examining the colour, moistness and degree of swelling of the vagina. To set up mating, females in oestrous (natural cycle) and females after hCG injection (study group) were placed with stud males (with proven fertility) for overnight. Mating was confirmed in the morning through observation of copulatory plug in the vagina (day 1 of pregnancy). Female mice were sacrificed and embryos were collected from ampulla of fallopian tube on day 1 (single cell stage) or day 2 (4-6 cell stage) or day 3 (8-12 cell stage) of pregnancy<sup>8</sup>. This variation in the time for collection of mouse embryos was adopted to distinguish effect of gonadotropins (best with early stage *viz.*, 1 or 2 cell stage) from errors during mitotic division (advanced stage embryo, in our case 8-16). For mosaicism study, it is also better to take more advanced stage embryo than 1-2 cell stage hence embryos of wide category day 1 to day 3 were taken. Embryos were graded morphologically immediately after collection as per criteria described by Hardarson *et al*<sup>9</sup>. It grades embryos into four categories based on size of blastomeres and percentage of cytoplasmic fragmentation *viz.*, Grade I (embryos with evenly sized blastomeres with or without fragments of <20%), Grade II (embryos with unevenly sized blastomeres with or without fragments of <20%), Grade III (embryos with 20-50% fragmentation and/or non-homogeneous cytoplasm and/or multinucleate blastomeres) and Grade IV (embryos with >50% fragmentation).

A total of 405 mouse preimplantation embryos of 2-16 cell stages in gonadotropins stimulated cycles (study group) and 111 mouse preimplantation embryos in natural cycles (control group) were obtained. Mouse embryos were washed several times with embryo

culture medium (Cook, India) at 37°C to remove any maternal cell under zoom dissecting microscope (Olympus, Japan). The mouse embryos thus obtained were transferred to silane/poly-l-lysine coated slides (Sigma, India) onto a pre-marked position and then zona-pellucida was dissolved using Tween20 (Promega, USA) - HCl for 3-5 min as per protocol described by Harper *et al*<sup>10</sup> under zoom dissecting microscope (Olympus, Japan). Embryo nucleus was then fixed in methanol:acetic acid (3:1 ratio) fixative, dehydrated in 70, 90 and 100 per cent ethanol series for 1 min each and air dried. Slides were then aged at 90°C for 90 min before subjected for FISH.

Mouse chromosome X, Y and 19 specific FISH probes (whole chromosome paint ready to use probes from Cambio, UK and bacterial artificial chromosome DNA probe clones from Open Biosystem, USA as well as Uniba Biologia, Italy) were used for the study. Bacterial Artificial Chromosome (BAC) clones were grown in Luria-Bertani (LB) medium overnight and probe DNA extracted as per PhasPrep BAC DNA extraction Kit (Sigma, India) and labelled by nick translation method (nick translation kit Roche, Germany).

Slides were then treated with solution containing pepsin (100 mg/ml; Sigma, India) in 0.01N HCl for 20 min at 37°C, rinsed twice in double-distilled water and once in PBS (phosphate buffer saline; Sigma, India). Slides were then fixed in solution containing 1 per cent paraformaldehyde (Sigma, India) in PBS for 10 min at 4°C, rinsed in PBS twice and once in double-distilled water. Slides then dehydrated through ethanol series (70, 90 and 100 per cent) for 3 min each and air dried before subjecting for FISH.

About 5 µl probe mixture (200-300 ng labelled each probe DNA in hybridization buffer consisting of 60 per cent formamide, 2X saline sodium citrate (SSC), 10 per cent dextran sulphate; Sigma, India) was then applied to the slides under a circular cover slip (11 mm in diameter). The probes and nuclear DNA are then co-denatured at 76°C for 6 min. Slides then hybridized overnight at 37°C in a hybridization block (FISH hybrite, Vysis Inc., USA). After hybridization cover slips were removed gently and slide immersed for 2 min in 50 ml of solution containing 0.4XSSC/0.3 per cent NP-40 (Sigma, India) at 73°C into a coplin jar followed by another wash of 2 min at room temperature in 50 ml of solution containing 2XSSC/0.1 per cent NP-40 into a coplin jar. Then slides were

dehydrated in 70, 90, 100 per cent ethanol series and air dried at dark. Slides then stained and mounted with cover slip containing 10 µl antifade (Vector, USA) containing DAPI (4',6-diaminidino-2-phenylindol; Sigma India) at concentration of 1 µg/ml. Excess of antifade was removed and slides viewed using Olympus BX51 microscope (Olympus, Japan) using 100 watt mercury bulb, 100X plane apochromatic objective and appropriate filter sets [single band pass filter for DAPI, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC)]. Images captured with the help of digital camera (Olympus DP11 & Camedia Master 1.2 software, Japan) in initial part of the study and FISH imaging system (Applied Spectral Imaging system & FISH view software version 4.5, Israel) in the later part of the study. Two rounds of FISH were performed, first dual colour FISH using chromosome X (FITC labelled, green; Roche, Germany) and chromosome 19 (TRITC labelled, red; Roche, Germany/Cy3 labelled, red, Amersham, UK) or chromosome X (green; FITC) and Y (red; Cy3/TRITC) and after visualization and interpretation second round of FISH *i.e.*, re-FISH with chromosome Y (red) or chromosome 19 FISH (red) after removing cover slip and washing in PBS.

*Statistical analysis:* All data derived from the study were plotted along with mean ± SD. All plots and statistical analyses were calculated using the Sigma Plot Software (Cranes Software International Ltd., Bangalore, India). Chi square test and Fisher exact test were used and  $P < 0.05$  was considered statistically significant. The statistical analyses were carried out using Statistical Package for Social Sciences (SPSS Inc., USA).

## Results

Detailed information on embryos including morphological grading obtained from both control and study group is represented in Table I. More number of embryos were retrieved from the right uterine tube.

Table II summarizes detail information on embryo FISH studied by chromosomes X, Y and 19 probes (Fig. 1). In control group, FISH results were interpretable in 66 embryos whereas representative figure in study group were 128. In study group there was one case of sex chromosome aneuploidy (XYY1919; Fig. 2) with 10 IU folligon group and no mosaicism whereas in control group, there were no aneuploidy or mosaicism. No effect was observed with dose or preparations of gonadotropins.

**Table I.** Details of embryos obtained from control and study groups

Parameters	Control group	Study group	5 IU Folligon	10 IU Folligon	15 IU Folligon	5 IU PMSG	10 IU Pergonal
Number of mice	19	25	7	9	3	3	3
Number of embryos obtained	111	405	78	154	70	28	75
Embryo/mice (mean $\pm$ SD)	5.8 $\pm$ 2.7	16.2 $\pm$ 10.8	11.1 $\pm$ 7.4	17.1 $\pm$ 10.4	23.3 $\pm$ 14.4	9.3 $\pm$ 7.5	37.5 $\pm$ 3.5
Left oviduct	46	176	30	65	34	12	35
Right oviduct	65	229	48	89	36	16	40
Morphology							
Grade I	109	401	75	153	70	28	75
Grade II	2	4	3	1	0	0	0
No of blastomeres	518	1918	364	874	396	134	150
Blastomeres/embryo	4.6	4.7	4.6	5.6	5.6	4.7	2
Interpretable FISH Results obtained (number of embryos)	66	128	26	53	21	7	21

PMSG, pregnant mare serum gonadotropin

**Table II.** Summary of FISH\* results

Parameters	Control group	Study group	5IU Folligon	10 IU Folligon	15 IU Folligon	5 IU PMSG	10 IU Pergonal
Male embryos	19	71	14	29	12	4	12
XY1919 (XY19 FISH)	14	04	4				
XYY1919 (XY19 FISH)	0	1	0	1	0	0	0
XY (XY FISH result only)	0	44	4	19	8	1	12
X1919 (X19 FISH result only)	0	22	6	9	4	3	0
Y (Y FISH result only)	5	0	0	0	0	0	0
Female embryos	14	57	12	24	9	3	9
XX1919 (XY19 FISH)	14	29	12	11	3	3	0
XX (XY FISH result only)	0	28	0	13	6	0	9
1919 (19 FISH result only)	33	0	0	0	0	0	0
Normal embryos	66	127	26	52	21	7	21
Abnormal embryo	0	1	0	1	0	0	0
XYY1919 (XY19 FISH)	0	1	0	1	0	0	0
Male: Female	19:14	71:57	14:12	29:24	12:9	4:3	12:9
Sex-ratio	1.36:1	1.25:1	1.16:1	1.2:1	1.3:1	1.3:1	1.3:1
P value		P=0.8	P=0.77	P=0.79	P=0.97	P=0.98	P=0.97
Total	66	128	26	53	21	7	21

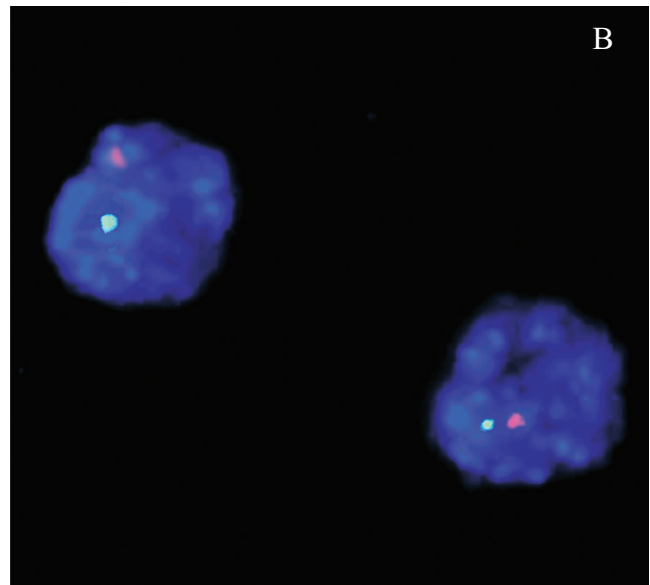
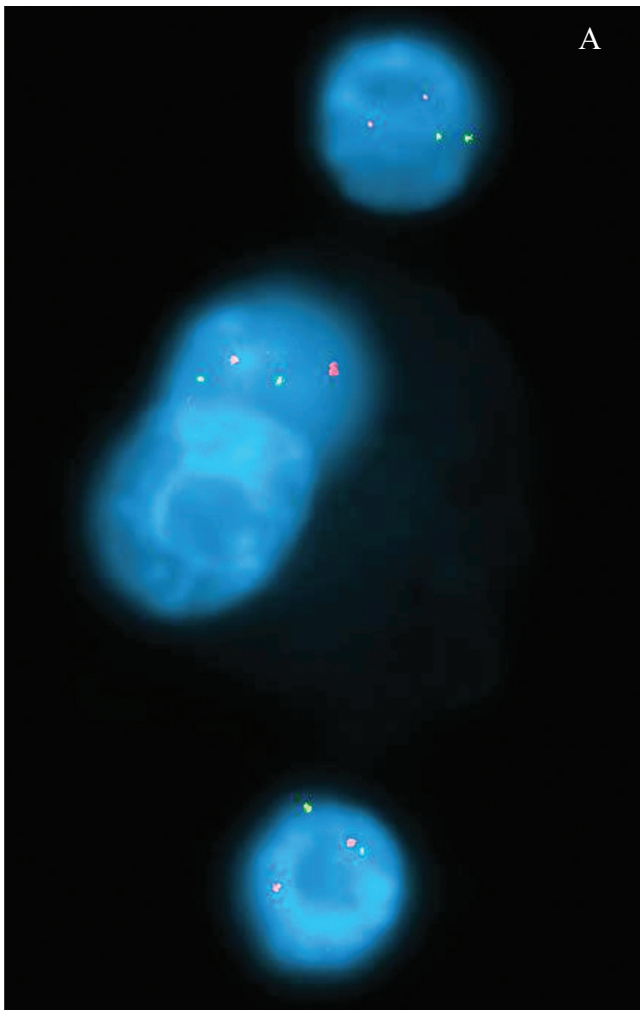
\*FISH experiments were done in 2 rounds; first round as either XY FISH or X19 FISH followed by second round as 19 or Y re-FISH; results of all 3 probes (chromosomes) in all embryos were not obtained due to various reason viz., failure of FISH (either at first round or at second round) or dislodgement/washed out of embryos mostly during second round of FISH i.e., re-FISH

There were 71 male and 57 female embryos in the study group (sex ratio of 1.25 M:1F). No significant variation was seen in sex ratio with dose or preparations of gonadotropins (Table II). In control group, sex ratio was interpretable in 33 embryos. Out of 33 embryos, 19 were male (XY1919/Y) and 14 were female (XX1919) thus giving a sex ratio of 1.36 M:1F. In other 33 embryos, 1919 chromosome pattern was observed and there was no signals for chromosome X or Y due to failure of XY FISH.

## Discussion

The present study was done to assess effect of gonadotropin (used for hyperstimulation protocol) on chromosome aneuploidy and chromosome mosaicism of mouse preimplantation embryos through FISH (X, Y and 19 probes) using whole embryos (all blastomeres).

Hansen *et al*<sup>5</sup> highlighted the potential risks of major birth defect (many are due to chromosome abnormality) in assisted reproduction (IVF and IVF-ICSI

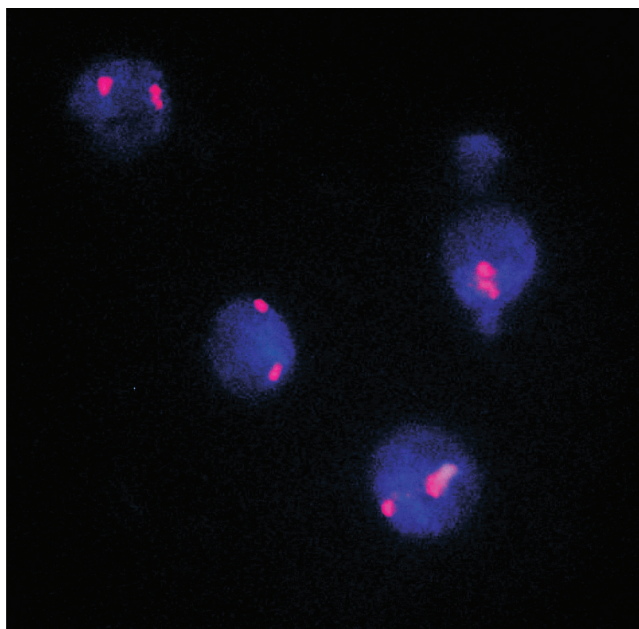


**Fig. 1.** (A) Three blastomeres from a 4 cell- stage embryo is showing two red signals of Ch 19 and two green signals of Ch X, one blastomere is not showing any signals (may be out of focal plane or non-hybridization). (B) Two blastomeres from a 2 cell stage embryo is showing one green (Ch X) and one red (Ch Y) signal *i.e.*, male embryo.

pregnancies) in comparison to naturally conceived pregnancies. Similarly, reduction of aneuploidy rate was also observed with milder ovarian stimulation protocol<sup>6</sup> indicating risk associated with gonadotropin usages. However, with human studies it is not possible to distinguish effect of drug (ovarian hyperstimulation) from various endogenous and exogenous factors *viz.*, infertility, maternal age, genetic heterogeneity (arising from outbred human population), *in vitro* condition, besides controls for comparison (difficulties in obtaining preimplantation embryos from natural cycles). Therefore, this study was carried out on mouse model to overcome difficulties with human studies.

All the interpretable embryos of the study and control groups were derived from group of identical (genetic, environment, diet, *etc.*) Swiss albino mice. All the embryos were of good quality (mainly grade 1) and only one embryo in the study group was found to have sex chromosome trisomy (XYY1919).

This indicated that gonadotropins used for inducing ovulation did not produce excess morphologically abnormal embryos as well as excess chromosome X, Y, 19 abnormality in mouse preimplantation embryos. However, this result should be interpreted with caution as only three chromosomes were studied in a small number of embryos. Interestingly, similar observation is also reported by Ma *et al*<sup>11</sup>. They found no significant effect of PMSG on the level of aneuploidy and structural abnormalities of 8-16 cell-stage embryos in the CD-1 mouse. Similarly, Fraser & Maudlin<sup>12</sup> did not observe any detectable differences in either *in vitro* gonadotropins stimulated embryos or *in vivo* spontaneously conceived embryos. However, a few studies have indicated that oocyte and embryo quality may be affected by high exogenous gonadotropin used for stimulation<sup>13,14</sup>. Above studies had shown significantly greater chromosomally abnormal embryos in the superovulated group than in the spontaneously ovulating group through conventional cytogenetics. This contradictory result could be due to variable susceptibility with gonadotropins due to genetic differences of study mice population rather than directly gonadotropins. This is also supported by several studies<sup>15,16</sup>. Wang & Hoog<sup>15</sup> proposed that abnormal karyotype of embryos results from absence of synaptonemal complex protein 3 (SCP3) and unlikely



**Fig. 2.** Chromosome Y FISH on four blastomeres derived from a 4 cell stage embryo is showing 2 copies of chromosome Y (2 red signals).

due to gonadotropins. In the absence of this protein meiotic chromosome segregation becomes defective thus promotes aneuploidy. Structural damage/change or absence in SCP3 in meiotic chromosomes impairs DNA recombination and checkpoint control in mammalian oocytes. Therefore, SCP3 is required for chiasmata formation for the structural integrity of meiotic chromosomes and absence of SCP3 promoted aneuploidy due to altered chromosomal structure and triggering non-disjunction of chromosome<sup>17</sup>. Akiyama *et al*<sup>16</sup> demonstrated that inhibition of histone deacetylation during female meiosis induces aneuploidy in mice. Based on above studies and results of our own study for the possible role of gonadotropins in the chromosome abnormalities it is possible that gonadotropins probably do not have direct effect in causing chromosome aneuploidy.

In this study skewed sex ratio (more male) was also observed in embryos however there was no effect of gonadotropin as similar skewing was observed in both the control and study groups. In one of the early studies of sexing mouse embryos by FISH, Gimenez *et al*<sup>18</sup> found a sex ratio close to the expected 1M:1F. This is in contrast to our findings skewed sex ratio was seen. Excess of males was found in both the groups, though the difference between the groups was not significant, and hence one can exclude any effect of gonadotropins. Similar findings were also reported

by Sakai and Endo<sup>19</sup>. The reason for skewed sex ratio in pre-implantation embryo is not known at present, however, it seems biological and may be related to fertilization preference by Y bearing spermatozoa<sup>20</sup>.

We also observed more embryos in right tube than the left both in control (58.5%) and study (56.5%) group. The exact reason for this difference is not known. However, similar observation was also noted by some other researchers in mouse<sup>21,22</sup> and in human<sup>23,24</sup>. Interestingly, some large human studies<sup>25,26</sup> with natural cycle also observed a frequency of right sided ovulation of about 55 per cent. Asynchrony in the activity of two ovaries is not unique to mouse or human, in fact in some species this may be more pronounced, *e.g.*, birds, whales, chinchillas. The mechanism by which the two ovaries differ in their activity is not known, however, it could be due to anatomical, vascular and embryonic developmental differences (gonads, kidney & adrenal) between sides<sup>27,28</sup>. The known difference between the right and left gonads in human lies in the anatomy of veins, left drains to left renal vein whereas right drains to inferior vena cava. This difference may be responsible for most varicocele in left side<sup>27</sup>. In women ovarian venous reflux and retrograde flow is common in left side causing congestion and varicosity on left side more often than right<sup>28</sup>. Venous stasis to left side may result in longer clearance of metabolites and subsequently diminished responsiveness in ovulatory cycles.

To conclude, gonadotropins used for ovarian stimulation had no direct effects in causing increase in chromosome X, Y and 19 aneuploidy, mosaicism and skewing of sex ratio in mouse model. However, this should be interpreted with caution as only three chromosomes were studied in a small sample of embryos. A large scale study with more FISH probes on a larger sample size need to be done to validate this preliminary observation.

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