Evolution of the meiotic prophase and of the chromosome pairing process during human fetal ovarian development

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BACKGROUND: Studies on human oocytes in prophase I are limited due to the difficulty in obtaining the sample. However, a complete study of meiotic prophase evolution and the homologue pairing process is necessary to try to understand the implication of oogenesis in the origin of human aneuploidy. METHODS: A complete analysis of meiotic prophase progression comprising the long developmental time period during which meiotic prophase takes place, based on the analysis of a total of 8603 oocytes in prophase I from 15 different cases is presented. The pairing process of chromosomes 13 and 18 is also described. RESULTS: The findings significantly relate for the first time the evolution of meiotic prophase to fetal development. Although for both chromosomes 13 and 18 a high pairing efficiency is found, pairing failure at the pachytene stage has been observed in 0.1% of oocytes. However, errors at the diplotene stage are substantially increased, suggesting that complete, premature disjunction of the homologues commonly occurs. Moreover, pre-meiotic errors are also described. CONCLUSIONS: Our findings show that homologous chromosomes pair very efficiently, but the high frequency of complete, premature homologue separation found at diplotene suggests that mechanisms other than the pairing process could be more likely to lead to the high aneuploidy rate observed in human oocytes.

Key words: aneuploidy/fetal development/human oocytes/meiosis/synapsis

Introduction

Gametogenesis implies the reduction of the chromosome complement to haploidy, to restore diploidy after fertilization. This process is accomplished by meiosis, in which the search for homology, recognition and exchange of genetic material occurs between homologous chromosomes.

In the human female, meiosis starts during the fetal stage. Therefore, samples are seldom available. For this reason, studies performed in human oocytes in prophase I have been scarce. However, the involvement of oogenesis in the origin of aneuploidy is crucial (Hassold and Hunt, 2001). Several researchers have studied the early meiotic stages in human female gametogenesis using classical approaches (Ohno et al., 1962; Baker, 1963; Blandau, 1969; Kurilo, 1981; Bojko, 1983; Speed, 1985; Garcia et al., 1987, Garcia et al., 1989), fluorescent in situ hybridization (FISH) (Cheng and Gartler, 1994; Cheng et al., 1995, 1998, 1999; Cheng and Nakatani-Cecchini, 2004) and, more recently, immunofluorescence (IF) (Barlow and Hultén, 1997; Hartshorne et al., 1999; Tease et al., 2002; Roig et al., 2004, Roig et al., 2005; Lenzi et al., 2005). These studies have allowed the observation of diverse female meiotic particularities which may be responsible for the high rate of chromosome non-disjunction found in human oocytes.

The main goal of this study is to analyse meiotic prophase over a long period of fetal developmental to characterize meiotic prophase timing in depth. Synapsis of chromosomes 13 and 18 has also been studied in human oocytes.

Materials and methods

Biological material

For the present study, 15 fetuses were used (Table I), all of them collected after legal interruption of pregnancy. Informed consent was obtained from the parents according to the Ethical Rules Committee of the Hospital Vall d’Hebron and Clínica Tutor Mèdica, Barcelona, Spain. Gestational age was calculated based on the last menstrual period, foot length and echography, when available.

Oocyte spreading

Ovaries were collected at the hospital in phosphate-buffered saline (PBS) with 1% penicillin–streptomycin (Gibco-Life Technologies, Paisley, UK) within the first 6h of delivery and transported within 30min to the laboratory. Under sterile conditions, ovaries were dissected from adjacent tissues and washed in PBS.

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Spreading of the oocytes was performed as previously described (Roig et al., 2005). In order to know the karyotype of the sample, in most of the cases (12 out of 15) a somatic culture of the stromal ovarian tissue was performed after extracting the oocytes, following the protocol set up in our laboratory (Roig et al., 2003).

**Fluorescent in situ hybridization**

Chromosome painting was performed in all cases using different commercial (Vysis, Groove, USA; and Cambio, Cambridge, UK) and non-commercial (kindly donated by Dr R.Stanon) whole-chromosome 13 and 18 probes and locus-specific identification (LSI) probe 13/21 (Oncor, Gaithersburg, MD). The hybridization protocol was performed as described in a previous, published study (Roig et al., 2005). DNA was counterstained applying an antifade solution (Vector Laboratories, Peterborough, UK) containing 0.1 µg/ml DAPI (4’,6-diamidino-2-phenylindole; Sigma, Tres Cantos, Spain).

Oocytes were substaged according to morphological criteria described in previous articles (Garcia et al., 1987; Roig et al., 2005). In summary, at leptotene, chromosomes start condensation and individualization. At the zygote stage, homologue pairing starts and thickening of the bivalents is visible where synapsis is completed. At pachytene, bivalents are completely paired, and thus they appear thicker than in the rest of the stages. Finally, at diplote, homologues separate and remain close only joined by the chiasmata.

**Microscopy and image analysis**

Slides were analysed in an Olympus AX70 fluorescent photomicroscope (Olympus Optical Co., Hamburg, Germany). Images were captured and produced by a Cytovision system (Applied Imaging, Sunderland, UK), and were processed using Adobe Photoshop for publishing purposes.

**Results**

**Ovarian culture reveals a euploid karyotype of the cases studied**

In all cases where an ovarian culture was set up (Table I), a euploid karyotype (46,XX) was obtained, validating these cases as chromosomally normal in order to be used as controls to study the normal meiotic progression and homologue synapsis in human oocytes in prophase I.

**Meiotic prophase progresses as the ovary develops**

In this study, fetal foot length was used as a developmental marker. For fetuses aged between 15 and 22 weeks of gestation, foot length ranged from 1.8 to 4.5 cm (Table I and Figure 1).

The proportion of meiotic prophase substages was scored for each case on the basis of morphological criteria described before (see above). In total, 8603 oocytes were analysed. Oocytes at the different stages of meiotic prophase were found in all cases, except for T10, where there was an absence of oocytes at the diplote stage (Figure 1). The results, shown in Figure 1, clearly demonstrate that the values obtained for each meiotic prophase stage can be significantly related to the fetal developmental stage. Thus, in this particular time frame, the percentage at leptotene significantly follows an inverse correlation to foot length (Fisher $F = 31.208; P = 0.0001$), leading to a decrease of the proportion at leptotene as the ovary develops. The pachytene stage significantly increases with time, adjusting to a cubic plot ($F = 12.544; P = 0.0007$), achieving a maximum percentage at the end of the period analysed, ~4.5 cm foot length. Diplote stage percentages also increase with time, but following a logarithmic curve ($F = 10.402; P = 0.0066$), suggesting that the maximum percentage of the diplote stage in meiotic prophase does not coincide with this time frame.

Zygote proportions are similar in all cases analysed; percentages range from 19 to 34%. However, zygote figures do not significantly adjust to any curve. Nevertheless, the best correlation is obtained using a quadratic curve ($F = 1.778; P = 0.2106$). According to this correlation, a maximum proportion of zygote cells is found when the fetus foot length is ~3 cm, coinciding with the decrease of the leptothe stage proportion and the increase of the pachytoe stage proportion.

**Chromosome 13 and 18 pairing distribution throughout meiotic prophase**

In cases T14, T10, V54, V56, V44 and V61, the pairing process of chromosomes 13 and 18 was analysed in a total of 4885 oocytes (Figure 2 and Table II).

For chromosome 13, at leptothe, 92% of the oocytes have two separate chromosomes (two chromosome 13 signals) (Figure 2A and B); the remaining 8% of the studied oocytes at this stage had an already paired bivalent 13 (one chromosome 13 signal).

At zygote, 41% of the oocytes had two separate chromosome 13s (Figure 2C), while the other 59% had an already paired or a still pairing bivalent 13 (one signal) (Figure 2D). At pachytene, almost all oocytes showed a bivalent 13 (Figure 2E). However, we found one oocyte out of 1231, representing 0.1% of the studied pachytenes, in which both chromosome 13s failed to pair. At diplote, all oocytes
studied had a desynapsing bivalent 13 (recorded as a single signal in Table II) (Figure 2F).

The chromosome 18 results were not statistically different from those obtained for chromosome 13, with the exception of the leptotene stage in which 86% of the analysed oocytes had two separated chromosome 18s, 13% an already paired bivalent 18 and, surprisingly, 0.6% had three chromosome 18s (Table II). These results are significantly different from those described before for chromosome 13 ($\chi^2 = 12.757; P = 0.002$). At zygotene, 37% of the oocytes had two separated chromosome 18s, while the majority (63%) had an already paired bivalent 18. At pachytene, 99.8% of the oocytes had a bivalent 18, while 0.2% failed to produce the bivalent. No trisomic oocytes were observed at this stage. At diplotene, 97% of the oocytes showed a desynapsing bivalent 18, and 3% had two separated chromosome 18s.

**Dynamics of the chromosome 13 pairing process**

Once chromosome 13 distribution through the meiotic prophase had been analysed, the dynamics of the chromosome 13 pairing

<table>
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<tr>
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<th>Pachytene</th>
<th>Diplotene</th>
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**Figure 1.** Meiotic prophase substage distribution for the different cases analysed. Correlations of (A) leptotene, (B) zygote, (C) pachytene and (D) diplotene percentages and foot length are shown. G.W = gestation weeks, F.L = foot length (measured in cm).
process during the zygotene stage were studied in depth. Thus, we classified how chromosome 13s behaved during the zygotene stage in all cases studied. Oocytes with two separated chromosome 13s (unpaired) were found (Figure 2C), some in which chromosome 13 synapsis had started (the synapsed portion of the chromosome was less than half; these were classified as ‘started pairing’ in Table III), some in which synapsis had almost finished (Figure 2D) and finally oocytes with a completely paired bivalent 13. The results, presented in Table III, show that there is no relationship between the pairing process and foot length or percentage of oocytes at the zygotene stage.

Discussion
Results covering most of the developmental frame in which human female meiotic prophase takes place are presented. The range of samples used in this study was defined by the accessible material. The low availability of fetuses younger than 15 weeks and the legal limitation existing in Spain to interrupt pregnancy within the first 22 weeks of gestation limited the developmental frame in which the study was performed.

Data presented in this study are extremely useful as a guide to understand how meiotic prophase evolves in the human ovary during fetal development. Moreover, this is the first study in which a statistically significant relationship between distribution of meiotic prophase substages in the ovary was related to the fetal developmental stage. In this sense, foot length, instead of gestational age, was found to be a better marker for fetal developmental stage (see above). Although most of the samples had pathologies, all fetuses were found to follow a normal fetal development (data not shown).

In the developmental frame studied herein, the proportion of leptotene tends to decrease from 49.5 to 12%. Absence of leptotene oocytes was not detected in any ovary, suggesting that initiation of meiosis starts before the 15th week of gestation, in agreement with previous studies where leptotene was first found at ~11–12 weeks of gestation (Ohno et al., 1962; Baker, 1963; Garcia et al., 1987). Our data also suggest that leptotene oocytes are still present beyond...
the 22nd week of gestation, as suggested before (Speed, 1985; Baker et al., 1996).

The proportion of zygotene oocytes tends to be similar at all developmental times studied, ranging from 20 to 35%, with a slight tendency to achieve a peak at around 20–21 weeks of gestation. For this reason, no significant statistical correlation was found between the zygotene stage proportion and the fetal developmental stage. On the contrary, the pachytene stage proportion was clearly found to increase with foot length. In young fetuses, the pachytene proportion was low (~25%); in older ones, the proportion reached 58.5%. These figures suggest that the first pachytene oocytes can be observed before 15 weeks of gestation, in agreement with what has been published (Ohno et al., 1962; Baker, 1963; Garcia et al., 1987). The results also suggest that the maximum pachytene proportion is not achieved in this developmental frame, in contrast to Baker (1963), who described this phenomenon at the fifth month of gestation (~21–22 weeks). This point would be clarified if studies in older fetuses could be performed. However, the need to collect samples as fresh as possible, because this is a critical factor to obtain informative preparations, prevents us from working with spontaneous abortions.

Surprisingly, diplotene stage oocytes were commonly found in the present study, even in young fetuses, in contrast to what had been published before (Ohno et al., 1962; Baker, 1963; Wallace and Hulten, 1985). The diplotene stage proportion in the time period analysed is always low (~10%), but a significant progression can be observed with the fetal developmental stage, increasing from 2 to 10%. Our results imply that the first diplotene oocytes would appear around weeks 15–16 of gestation, in agreement with a previous study (Garcia et al., 1987), but in contrast to other studies in which the first diplotene oocytes were seen at weeks 17 (Baker, 1963), 18 (Wallace and Hulten, 1985) and 19 of gestation (Speed, 1985).

Results obtained in this study and in the other published studies (Ohno et al., 1962; Baker, 1963; Speed, 1985; Wallace and Hulten, 1985; Garcia et al., 1987; Cheng and Gartler, 1994) show a high heterogeneity of meiotic prophase evolution related to fetal age. This can be easily explained if we take into account that most of the studies are performed on therapeutically aborted fetuses, which may have a developmental delay due to their pathology. However, if an objective parameter that describes fetal developmental progression, such as foot length, is used, a better statistical correlation between meiotic prophase substages and fetal development can be achieved. Nevertheless, inter-individual differences have been clearly observed in this study, as demonstrated by the different meiotic prophase stage proportions found in cases V55 and V64, or cases V84 and V67, which have the same foot length (Figure 1).

The pairing process of chromosome 13 was studied in detail because it causes the third most common autosomal trisomy in live births, and a complete analysis of this chromosome pairing process during meiotic prophase had not been performed previously. The meiotic pairing process of chromosome 18, however, has been published (Cheng et al., 1995); thus, this chromosome was chosen as a marker in order to compare data obtained in our laboratory with previously published data.

The pairing process of chromosomes 13 and 18 demonstrates the existence of a high pairing efficiency, coinciding with what has been reported in studies that used FISH (Cheng and Gartler, 1994; Cheng et al., 1998), but in contrast to other observations in which synaptonemal complexes (SCs) were analysed (Speed, 1985). Differences found between these studies may be due to the different technical approaches used.

For both chromosomes 13 and 18, pairing was observed to start during the leptotene stage (Table II). This phenomenon, which has already been described for chromosome 18 (Cheng et al., 1995), confirms a recent study that found bouquet topology (known to facilitate homologue synopsis) at the leptotene/zygotene transition (Roig et al., 2004). Nonetheless, Cheng et al. (1995) found a large proportion (48%) of leptotene stage oocytes with an already paired bivalent, which substantially differs from our data (13%).

The proportion of paired chromosome 18 s at the zygotene stage found in this study also differs from published results (63 and 93%, respectively). All of these differences may be attributable to inter-individual differences, but different substaging criteria cannot be excluded.

The pairing process of chromosome 13 seems to be analogous to that described for chromosome 18. However, a lower proportion of paired chromosome 13 s at leptotene was found when compared with chromosome 18 (8 and 13%, respectively; Table II). This may be related to the different length of chromosomes which would enable the shorter chromosome 18 to pair faster than the longer chromosome 13.

In any case, this different pairing speed observed does not affect the pairing efficiency of any of the chromosomes studied. Pairing anomalies found for chromosomes 13 and 18, such as pachytene oocytes with unpaired chromosomes, are not statistically different. These are present in 0.13% of the total number of oocytes analysed for both chromosomes (n = 2352). These numbers are consistent with results found by Cheng et al. (1994, 1998) for the X chromosome and for

<table>
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<th>Finished pairing (%</th>
<th>Started pairing (%</th>
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chromosome 21, but differ from results obtained for chromosome 18 (0%), although this could be related to the low number of oocytes analysed by Cheng et al. (1995) as compared with this study.

Surprisingly, anomalies found at the diplotene stage (two out of 149 studied diplotene stage oocytes, representing 1.3%) are significantly higher than at pachytene. This figure includes oocytes that have failed to pair their homologues and oocytes in which premature homologue disjunction has occurred, either by failure to establish chiasmata or by the precocious resolution of chiasmata.

The presence of two univalents implies independent segregation of the homologues at the first meiotic division which may lead to unbalanced oocytes.

The 10-fold higher pairing anomaly rate found at the diplotene stage compared with that found at the pachytene stage means that premature homologue disjunction at diplotene is a more important factor in terms of producing abnormal chromosome segregation during meiosis rather than bivalent formation during the zygote and the pachytene stages. In fact, in the male, the most common anomalies limited to the germ cell line are the complete (presence of univalents) or partial (desynaptic bivalents) separation of homologues at diplotene (Egozcue et al., 2005, in press).

The anomaly rate found at the diplotene stage is similar to the frequency of aneuploid pregnancies found in young mothers (~2% in women under the age of 25; Hassold and Hunt, 2001), suggesting that this phenomenon, observed in the present study, may be an important factor in promoting aneuploidy in humans.

Bivalent 18 was found to be more prone to complete disjunction at the diplotene stage than bivalent 13 (Table II). This may be due to the fact that the long arms of the acrocentric chromosomes (13, 14 and 15) typically have two chiasmata (the cytological image of a crossing-over). Moreover, these chromosomes have a higher recombination frequency than similar sized non-acrocentric chromosomes (reviewed by Lynn et al., 2004). This fact supports previous findings leading to the conclusion that the high aneuploidy rate found in human oocytes seems to be attributable to recombinational errors rather than to an inefficient homologue pairing process.

In fact, our data suggest that pairing errors seem to affect all chromosomes similarly, while some chromosomes seem to be more susceptible to recombinational errors, as has already been proposed by Lynn et al. (2004).

Surprisingly, we found four leptotene stage oocytes (0.6%) with three chromosome 18 s. The presence of aneuploid oocytes in euploid fetuses has also been observed for the X chromosome (Cheng and Gartler, 1994). Nevertheless, this phenomenon was not observed for chromosome 13. The extra copy of chromosome 18 seems to come from a premeiotic non-disjunction, and suggests an increased tendency for non-disjunction for chromosome 18 as compared with chromosome 13. This excess could indicate that cells with a trisomy 18 are subject to a less negative selection than cells with a trisomy 13, which agrees with the milder symptomatology presented in Edward’s syndrome (trisomy 18) compared with Patau’s syndrome (trisomy 13).

The results from the dynamics of the chromosome 13 pairing process show that pairing occurs quickly, as demonstrated by the preferential finding of oocytes with unpaired or completely paired chromosome 13 s instead of bivalents in which the pairing process is underway. In this sense, we have not been able to relate the dynamics of the chromosome 13 pairing process to either foot length or percentage of oocytes at the zygote stage, suggesting that the pairing process of chromosome 13 may be related to other factors. More studies should be carried out in order to clarify these questions.

The results obtained in this study show that chromosomes pair efficiently. The pairing process in human female oocytes has also been studied in abnormal cases where there was the presence of an extra chromosome (Speed, 1984; Cheng et al., 1995, Cheng et al., 1998; Roig et al., 2005) or reorganized genomes (Cheng et al., 1999). These studies found that even in these circumstances, homologues complete the pairing process, some of them with an efficiency similar to that described here (Roig et al., 2005). Our findings, together with the data obtained in aneuploid oocytes, suggest that pairing process fidelity seems sufficient to ensure bivalent formation at the pachytene stage. The 10-fold higher anomaly rate found at the diplotene stage suggests that complete, premature homologue disjunction may play an important role in the origin of the high aneuploidy rate observed in human oocytes (Hassold and Hunt, 2001). More studies on human female oocytes should be performed to better understand the cause of this problem.

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