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Unusual chromosome cleavage dynamic in rodent neonatal germ cells

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Abstract At the metaphase/anaphase transition in the mouse and rat male germ lines during the perinatal period, sister centromeres separate before sister chromatids. This gives the chromosomes an unusual appearance that resembles the premature centromere division described in some human pathological conditions such as Roberts syndrome. At the same period, there is also an unusual pattern of DNA methylation, with strongly demethylated heterochromatin and methylated euchromatin. This suggests that chromosome DNA methylation may modulate chromatid and centromere splitting, without altering normal chromosome segregation.

Introduction

Centromeric regions of metaphase chromosomes are usually observed as primary constrictions containing tightly attached sister centromeres, whereas sister chromatids can exhibit various stages of splitting. The strong cohesion of centromeres until initiation of anaphase is considered to be a prerequisite for normal chromosome segregation at mitosis. In some conditions, the temporal difference between chromosome arm and centromere cleavage is altered, leading to so-called premature centromere division (PCD). Premature centromere division has been found to occur in a few rare syndromes (including Roberts syndrome), in cancer cells, or after genotoxic treatments (Jabs et al. 1989; Van Den Berg and Francke 1993; Hsu et al. 1998; Kawame et al. 1999; Major et al. 1999; Barbosa et al. 2000). It has been associated with chromosome instability, aneuploidy, and altered duration of metaphase. This suggests that PCD favors abnormal mitotic segregation. Nevertheless, precocious centromere separation has been shown to occur naturally in some organisms, such as budding yeast, diatoms, and the crustacean *Ulophysema öresundense*, suggesting that sister centromere cohesion is not a prerequisite for normal chromosome segregation (Nasmyth et al. 2000). This has also been demonstrated in vertebrates, using a laser microbeam to sever the centromere between the kinetochores of prometaphase chromosomes of PtK1 cells: arm cohesion between sister chromatids was sufficient to support bipolar orientation and congression (Rieder and Cole 1999).

A frequent characteristic of mammalian chromosomes is the presence of highly methylated DNA in their heterochromatic regions. This can be visualized by using an anti-5-methylcytosine (anti-5mC) antibody on metaphase chromosomes (Barbin et al. 1994). In vitro, DNA methylation can be altered by the demethylating agent 5-aza-deoxycytidine (5-aza-dC). In vivo, the pattern of DNA methylation spontaneously varies from tissue to tissue and in a given cell type with time and physiological conditions. These epigenetic variations are quantitatively significant enough to be directly detected on chromosomes by immunocytochemistry (Kokalj-Vokac et al. 1993; Bernardino et al. 1996; Rougier et al. 1998; Vilain et al. 2000).

We have characterized the changes in the DNA methylation pattern of testicular cells during mouse perinatal spermatogenesis (Coffigny et al. 1999; Bernardino et al. 2000). Briefly, metaphase chromosomes from somatic cells (mostly Sertoli cells) keep the same morphological characteristics and DNA methylation pattern during this period. Their chromosomes have short, fuzzy, non-cohesive and weakly methylated arms, but strongly cohesive and methylated centromeric regions. In contrast, the DNA methylation pattern of germ cells changes extensively during the neonatal period. It is characterized by overall strong DNA methylation that occurs a few days before birth in G0/G1-arrested cells. At

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birth, germ cells resume their cycle and the first mitoses appear at 1 day post-partum. Their chromosome morphology and DNA methylation are quite different from those of Sertoli cells. Chromatids are hypermethylated, long, well delineated and strongly cohesive, whereas centromeric regions are nonmethylated, decondensed and non-cohesive. With successive cell divisions, the centromeric DNA progressively becomes more methylated while the DNA hypermethylation of chromatids is semi-conservatively lost. This results in a decrease by half in the number of strongly labeled chromatids per cell generation, allowing us to estimate the number of cell divisions that have occurred since 'birth' for each analyzed cell.

In this study, we improved mitotic germ cell preparation and found that neonatal germ cells have specific chromosome segregation dynamics with sister centromeres separating before sister chromatids. Because the variations in chromosome morphology of germ cells correlate with the variations in their DNA methylation pattern, we propose that DNA methylation could be involved in chromosome cohesion.

Materials and methods

Animals

The NMRI mice were bred and mated in our animal facility. Pregnant rats were purchased from IFFA CREDO and WMP pregnant mice were kindly provided by J.P. Guénet (Institut Pasteur, Paris).

Testicular cell chromosome preparations

Testicular cells were obtained from pools of testes from three litters from 2 to 12 days (mouse) and 5 to 9 days (rat) after birth. Briefly, testes were placed in a plastic dish filled with PBS in which the albuginea tunica was carefully removed. Testes were then incubated in 0.5 mg/ml collagenase I (Life Technologies) for 10 min at 28°C and steadily gently shaken. The cells were washed in HBSS buffered with 20 mM HEPES. Chromosome preparations were made with or without standard hypotonic shock (0.075 M KCl) using our standard protocol (Bernardino et al. 2000).

Anti-5-mC immunostaining

Immunostaining with anti-5-mC antibody was performed according to Barbin et al. (1994) with minor modifications described in Bernardino et al. (2000).

In vitro and in vivo colcemid treatment of 2 day old mouse testes

For in vitro treatment, testes from two mice were drawn and incubated for 2 h in DMEM/F12 medium supplemented with glutamine (2 mM), HEPES (5 mM), and glucose (0.03%) in the presence or absence of colcemid (0.04 μg/ml). For in vivo treatment, colcemid (0.6 mg/kg) or PBS (control) was injected intraperitoneally 2 h before sacrifice. Each experiment was repeated twice.

Scoring of splitting

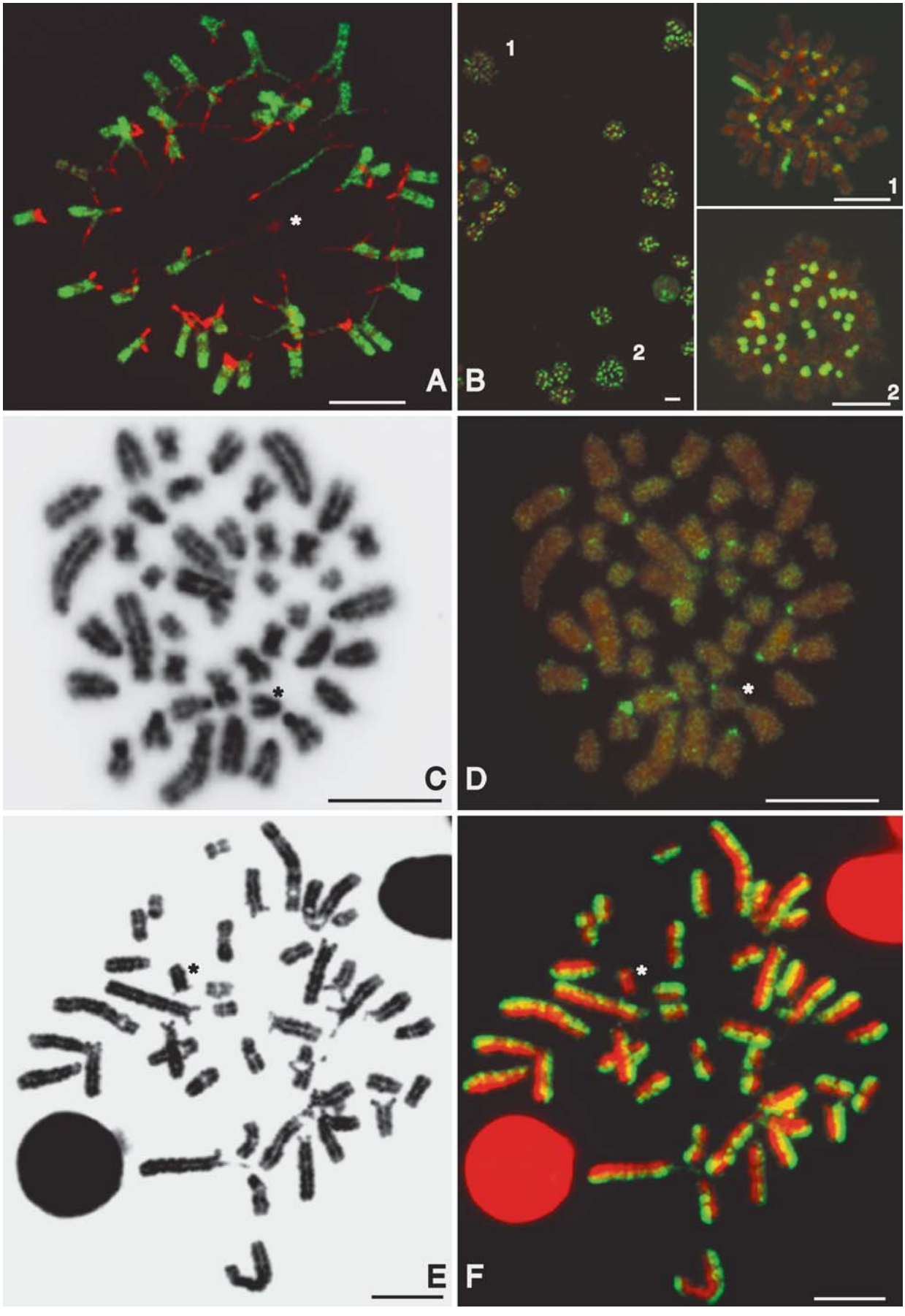
In a given metaphase plate, splitting could involve a variable number of chromosomes and, to a variable extent, from part of the heterochromatin to all of the heterochromatin plus part of the euchromatin. When only parts of the heterochromatin were involved, it was not possible to be certain that centromeres were affected; in this case cells were considered to exhibit heterochromatin splitting (HS). When all heterochromatin of at least one chromosome was completely split, the centromere was necessarily involved, and the cell was considered to exhibit PCD. Most generally, PCD and HS involved many or all chromosomes of a given cell. We use the term PCD, in spite of the fact that the observed phenomenon may correspond either to premature centromere division or delayed chromatid splitting.

Results

Mouse cells

When standard chromosome preparation methods were used, PCD and HS were occasionally observed in germ cell metaphases, contrasting with the strong cohesion of euchromatic sister chromatids. Neonatal germ cells (gonocytes), which possess a large nucleus (Nagano et al. 2000), are highly sensitive to hypotonic shock. Thus, we tried to improve our preparations by suppressing the hypotonic shock. This dramatically increased the mitotic index. In contrast, with Sertoli cells, germ cells exhibited well-spread metaphases, and we observed that almost all of them exhibited either HS or PCD. Their occurrence in relation to the number of cell cycles after birth indicated that PCD was significantly more frequent in germ cells undergoing their first two divisions than after more divisions after birth (χ^2=62; ν=2; P<0.0001; data from Table 1). Premature centromere division gave a very unusual morphology to metaphase chromosomes, whose

Fig. 1 Mouse (**A**, **B**) and rat (**C**–**F**) testicular cell metaphases. All metaphase spreads have been successively stained with 4',6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml) and immunostained with anti-5-methylcytosine (anti-5mC) antibody, revealed with a fluorescein isothiocyanate (FITC) conjugated anti-mouse antibody (*green*) and counterstained with propidium iodide (5 μg/ml) (*red*). **A** Cell that has undergone one cell cycle after birth, with elongation and extensive splitting of the centromeric regions (*red*). **B** *Left* low magnification exhibiting germ (*1*) and Sertoli cell (*2*) at metaphase on the same field, showing differential staining of their pericentromeric regions with anti-5mC antibody; *right*, the same germ (*upper insert*) and Sertoli cells (*lower insert*) at a higher magnification. In the germ cell that has undergone seven divisions, two chromosomes remain hemi-hypermethylated. Compared with **A**, pericentromeric regions become methylated but their staining is heterogeneous and their intensity of staining is lower than in the Sertoli cell. **C, D** Rat Sertoli cell exhibiting cohesive centromeric regions after DAPI staining (**C**) and stronger DNA methylation status than the chromatids (**D**). Note the diffuse and faint staining by anti-5mC antibody around the chromatids in Sertoli cell, compared with the well-delineated staining of germ cell chromatids. **E, F** Rat germ cells that have undergone two cell cycles after resumption from their quiescent period: all chromosomes exhibit a single hypermethylated chromatid. The premature centromere division (PCD) is more clearly detected after DAPI (**E**) than after immunostaining (**F**). The DAPI staining is shown as a negative. (* Y chromosome) *Bar* represents 10 μm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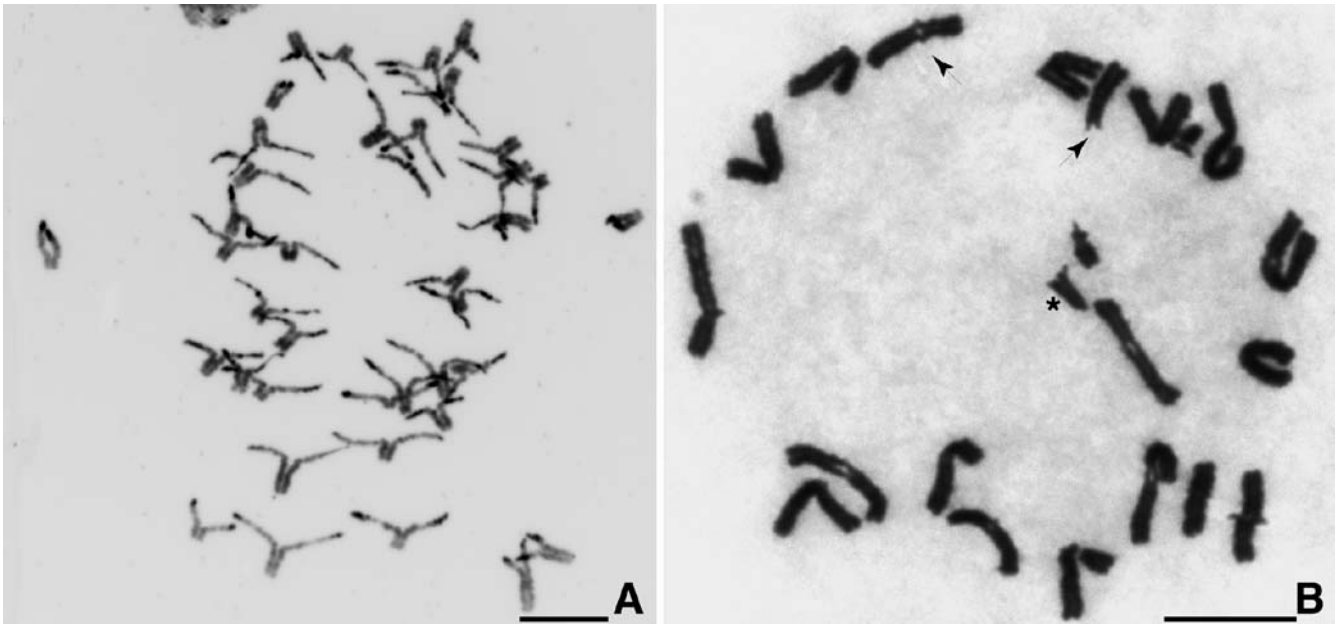

Fig. 2A, B Mouse germ cells exhibiting PCD. **A** First division metaphase of NMRI germ cell stained with DAPI (0.1 μg/ml) exhibiting extensive splitting of pericentromeric regions (*dark*) and adjacent regions. The DAPI staining is shown as a negative. **B** Second division metaphase of germ cell of WMP mouse stained with 1.5% Giemsa. Metacentric (bi-armed), acrocentric chromosomes (*arrows*) and the Y (*) exhibit PCD. *Bar* represents 10 μm

cohesion was ensured by their arms only (Fig. 1A). In the most advanced stages of splitting, cohesion was even limited to telomeric regions opposite to centromeres (Fig. 2A). Except for this centromere to telomere gradient, there was no apparent order in chromatid splitting, homologous chromosomes often exhibiting different appearances. Chromosome Y chromatids, which are the least rich in 5-mC, were recurrently among the less cohesive (Fig. 1A) and frequently completely separated. Occasionally, demethylated pericentromeric regions were extensively elongated and associated with those of other chromosomes (Fig. 1A). When chromatid hypermethylation was lost and heterochromatic centromeric regions became more methylated, the proportion of cells with PCD decreased. Although their chromatids were separated, neither PCD nor HS were observed in somatic cells, whose centromeric DNA was always strongly methylated (Fig. 1B).

In some experiments, we treated testicular cells with colcemid for 2 h. It very significantly decreased the proportion of cells with HS or PCD in both in vivo and in vitro conditions (Table 2). In vivo experiments with colcemid were less reproducible than in vitro, which can be explained by the heterogeneous quantity of colcemid delivered to the animal testes after intraperitoneal injection. Nevertheless, within each experiment, the decrease in HS and PCD after colcemid treatment was very significant, compared with untreated mice: in vitro (experiment a) χ^2=80.5, ν=1, P<0.0001; (experiment b) χ^2=89, ν=1, P<0.0001; in vivo (a) χ^2=54, ν=1, P<0.0001;

Table 1 Counts of germ cells with and without premature chromosome division (PCD). The number of metaphases with and without heterochromatin splitting was recorded after Giemsa staining. For the χ^2 test, we compared metaphases with no cleavage, with partial (HS) or complete (PCD) heterochromatin splitting

Number of cell cycles	No splitting	HS	PCD	Total cells analyzed
1 or 2	57	239	116	412
>3	100	232	28	360

Table 2 Effect of a classical 2 h colcemid (0.04 μg/ml in vitro or 0.6 mg/kg in vivo) treatment on detection of premature heterochromatin splitting. Counts of cell metaphases with or without HS and PCD of chromosome pericentromeric regions after staining with 1.5% Giemsa are shown. Two independent experiments (a, b) were performed

		Experiment	No splitting	Splitting (%)
In vitro	Control	a	13	51 (79.7)
		b	7	39 (84.7)
	With colcemid	a	92	11 (10.7)
		b	44	7 (13.7)
In vivo	Control	a	22	75 (77.3)
		b	10	55 (84.6)
	With colcemid	a	35	3 (7.8)
		b	63	30 (32.2)

(b) $\chi^2=85$, $v=1$, $P<0.0001$ (data from Table 2). This strongly suggests that detection of heterochromatin splitting is related to attachment of centromeres to the mitotic spindle.

Next, we analyzed neonatal germ cells from wild mice (WMP) with multiple metacentrics resulting from Robertsonian translocations (Zornig et al. 1995). Again PCD and HS were recurrently observed (Fig. 2B), but more frequently on acrocentrics than metacentrics (data not shown).

At all stages, DNA methylation of heterochromatic regions was less intense in germ cells than somatic cells. For instance, at the 6–7th division after birth, whereas heterochromatin of germ cells had been progressively methylated, heterochromatic regions of Sertoli cells were much more intensely labeled (Fig. 1B). In contrast, their euchromatin was less methylated than that of germ cells, which had lost their hypermethylation.

Rat cells

To check that HS and PCD are not limited to mouse chromosomes, which have large amounts of juxtacentromeric heterochromatin, we investigated male germ cells from the rat, which possesses many bi-armed chromosomes with smaller amounts of heterochromatin. As in the mouse, rat germ cells undergo the same DNA hypermethylation of euchromatin before birth, during their cell cycle arrest while their centromeric heterochromatin remains hypomethylated (Moreno et al. 2001). At 4–5 days post-partum, when germ cells resume their cycle, their chromosomes exhibit the same characteristics as in the mouse. Chromosomes of Sertoli cells were globally found to be weakly methylated except at their centromeric regions, which were highly cohesive and methylated (Fig. 1C, D). Heterochromatin splitting and PCD only involved germ cell chromosomes and more frequently acrocentrics than metacentrics (Fig. 1E, F). Premature centromere division occurred less frequently (and/or intensely) than in mouse metaphases, in which all chromosomes are acrocentric, but this might also be a consequence of differences in the composition of centromeric heterochromatin.

Discussion

We showed that sister centromere splitting recurrently occurs before that of chromatids in the first divisions of neonatal germ cells in both the mouse and rat. In the same preparations, this was never observed in testicular somatic cells whose DNA methylation pattern is exactly the reverse of that of germ cells. The appearance of germ cell chromosomes was similar to that of somatic cells in some pathological situations, leading to so-called premature centromere division (PCD, Van Den Berg and Francke 1993). Thus by analogy, we called the phenomenon PCD, although it might equally represent delayed sister chromatid splitting. Premature centromere division was less frequently observed in bi-armed than in acrocentric chromosomes. This was not strictly dependent on the DNA composition and quantity of centromeres since this difference was observed in both mouse, with or without Robertsonian translocations, and rat chromosomes. Moreover, the mouse Y chromosome also exhibits PCD, although neither minor nor major satellite DNA sequences, which are major components of pericentromeric regions in autosomes (Pardue and Gall 1970), are present.

A 2 h colcemid treatment, currently used in somatic cells to increase the cell mitotic index, drastically decreased the detection of PCD. This suggests that detection of PCD depends on the integrity of the mitotic spindle and the opposing traction forces exerted on the kinetochores. Heterochromatin elongation and associations were occasionally observed between non-homologous chromosomes in neonatal mouse germ cells. In human pathologies and in cultured cells treated with 5-aza-dC, such heterochromatin stretches and associations have been described and were correlated with a strong demethylation of these regions, which may promote chromosome rearrangements (Viegas-Pequignot and Dutrillaux 1976; Almeida et al. 1993; Jeanpierre et al. 1993; Tuck-Muller et al. 2000; Vilain et al. 2000). Thus a parallel may exist between these elongations/associations of hypomethylated heterochromatin observed in neonatal germ cells and the tendency of mouse karyotypic evolution to occur at a fast rate, principally via Robertsonian translocation intermediates (Britton-Davidian et al. 2000).

While in both mouse and rat chromosomes, weak DNA methylation is associated with lack of cohesion or premature splitting of either sister chromatids or sister centromeres, strong DNA methylation correlates with strong and/or prolonged cohesion. This is observed when comparing Sertoli cells with germ cells that have undergone one or two divisions after resumption of their cell cycle. It is also observed within germ cells along their divisions, for which a parallel exists between DNA methylation and chromosome cohesion. Considering the juxtacentromeric regions, it seems that there is a threshold of DNA methylation required for sister centromeres to separate after chromatids. This may also be valid for euchromatin because the strong chromatid cohesion observed in metaphases of the first two generations of germ cells is lost as the hypermethylation disappears. When all germ cell chromatids are no longer hypermethylated, chromatids become less cohesive. Consequently, their chromosomes become less easily distinguishable from those of Sertoli cells after simple Giemsa staining. Thus, the DNA methylation status of juxtacentromeric heterochromatin may be an important factor that influences the centromere splitting dynamic, but chromatid DNA hypermethylation may be an additional feature that favors PCD appearance and an unusual chromosome segregation mechanism by strengthening chromatid cohesion. In addition, we observed asynchrony of chromosome segregation, whereas the metaphase/anaphase

transition is assumed to be rapid. This suggests that this transition takes an unusually long time in this cell type, which would also explain the high rate of metaphase harvesting in the absence of a colcemid block.

Following alteration of DNA methylation status after 5-aza-dC treatment, dramatic and complex changes in chromosome compaction are induced in cultured lymphocytes (Flagiello et al. 2002). Thus both chromosome condensation and cohesion may be modulated by DNA methylation in neonatal testicular cells. This leads to the morphological differences observed between Sertoli and germ cells and also between germ cells at first and further divisions after birth. Premature centromere division was observed in vitro, after demethylating treatment of mouse cells in culture (Rodriguez et al. 2001). We also obtained a tenfold increase in the rate of PCD after two different demethylating treatments in a human breast cancer cell line (unpublished data). However, 5-aza-dC treatment induced neither PCD nor HS in cultured lymphocytes.

It has been suggested that PCD may be linked with some pathologies and aneuploidies. In Roberts syndrome, detection of PCD has been routinely used as a diagnostic criterion (Van Den Berg and Francke 1993). However, no link has been established with other cytological features present in this syndrome, such as delay in metaphase progression and anaphase abnormalities (Van Den Berg and Francke 1993). Barbosa et al. (2000) hypothesized that asynchrony in the time of replication of centromeric DNA may alter cohesion at this location but this remains to be demonstrated.

Structural abnormalities of heterochromatin have been described in lymphocytes from patients with ICF syndome (Jeanpierre et al. 1993; Tuck-Muller et al. 2000). DNA hypomethylation of these regions has been correlated with chromosome rearrangements such as sister chromatid exchanges, but neither PCD nor HS has been described. Thus, other parameters in addition to alteration of DNA methylation may be involved in the PCD phenotype.

An hypothesis for a direct relationship between DNA methylation and centromere cohesion would be that the binding of centromeric proteins and/or cohesin may be sensitive to DNA methylation. Mitchell et al. (1996) reported that 5-aza-dC treatment of mouse somatic cells could induce a redistribution of the centromeric protein CENP-B within the pericentromeric region. This suggests that DNA demethylation is able to displace some proteins, particularly centromeric proteins. It would be interesting to localize centromeric proteins in both Sertoli and neonatal germ cells, which exhibit opposite DNA methylation status of their centromeric regions. Interestingly, in *Drosophila*, the absence of the centromeric protein Mei-s332, which is not lethal, has been linked to PCD, and its overexpression decreased the frequency of PCD (LeBlanc et al. 1999). Recent studies on fission yeast have shown that protein Swi6/HP1, specifically interacting with heterochromatin, is crucial for centromere cohesion (Bernard et al. 2001) through the recruitment of the cohesin subunit Psc3 (Nonaka et al. 2002). In 10% of mouse embryonic cells lacking PTTG (a *Xenopus* securin homolog), PCD was observed, which suggests that proteins involved in the regulation of sister chromatid cohesion may also be involved in regulation of sister centromere cohesion (Wang et al. 2001).

In conclusion, rodent neonatal testicular cells, which possess unusual DNA methylation characteristics, are a good model in which to study chromatin structure and cohesion of both hetero- and euchromatin, and the possible role of DNA methylation in these processes.

We propose that, in the rodent neonatal testis, chromosomal protein interactions with chromatin and/or the mitotic spindle are modulated either directly by DNA methylation status, or via DNA methyl binding proteins. The characterization of the chromosomal protein content of neonatal testicular cells will help to explain this unusual chronology between sister chromatid and centromere splitting, and its possible consequences for the mode of chromosome segregation.

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