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# Nuclear organisation of sperm remains remarkably unaffected in the presence of defective spermatogenesis

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**Abstract** Organisation of chromosome territories in interphase nuclei has been studied in many systems and positional alterations have been associated with disease phenotypes (e.g. laminopathies, cancer) in somatic cells. Altered nuclear organisation is also reported in developmental processes such as mammalian spermatogenesis where a “chromocentre” model

is proposed with the centromeres and sex chromosomes repositioning to the nuclear centre. The purpose of this study was to test the hypothesis that alterations in nuclear organisation of human spermatozoa are associated with defects upstream in spermatogenesis (as manifest in certain infertility phenotypes). The nuclear address of (peri-) centromeric loci for 18 chromosomes (1–4, 6–12, 15–18, 20, X and Y) was assayed in 20 males using established algorithms for 3D extrapolations of 2D data. The control group comprised 10 fertile sperm donors while the test group was 10 patients with severely compromised semen parameters including high sperm aneuploidy. All loci examined in the control group adopted defined, interior positions thus providing supporting evidence for the presence of a chromocentre and interior sex chromosome territories. In the test group however there were subtle alterations in the nuclear address for certain centromeres in individual patients and, when all patient results were pooled, some different nuclear addresses were observed for chromosomes 3, 6, 12 and 18. Considering the extensive impairment of spermatogenesis in the test group (evidenced by compromised semen parameters and increased chromosome abnormalities), the observed differences in nuclear organisation for centromeric loci compared to the controls were modest. A defined pattern of nuclear reorganisation of centromeric loci in sperm heads therefore appears to be a remarkably robust process, even if spermatogenesis is severely compromised.

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### Abbreviations

|                    |                                       |
|--------------------|---------------------------------------|
| DNA                | Deoxyribonucleic acid                 |
| ICSI               | Intracytoplasmic sperm injection      |
| OAT                | Oligoasthenoteratozoospermia          |
| FISH               | Fluorescence in situ Hybridisation    |
| PBS                | Phosphate-buffered Saline             |
| SSC                | Saline sodium citrate                 |
| ddH <sub>2</sub> O | Double-distilled water                |
| DAPI               | 4',6-diamidino-2-phenylindole         |
| 3D                 | Three dimensional                     |
| 2D                 | Two dimensional                       |
| NDRP               | Not discernible from a random pattern |

### Introduction

The nucleus of any eukaryotic cell is highly compartmentalised with chromosomes occupying distinct nuclear territories at preferred locations (Cremer and Cremer 2001; Parada and Misteli 2002). This arrangement of interphase chromosome territories contributes to a functional nuclear landscape in which specific functions are provided within discrete nuclear compartments (Foster and Bridger 2005; Fraser and Bickmore 2007; Lanctot et al. 2007; Dundr and Misteli 2010; Rouquette et al. 2010; Schoenfelder et al. 2010; Pederson 2011; Rajapakse and Groudine 2011; Spector and Lamond 2011). Hence, the dynamic spatio-temporal organisation within interphase nuclei, so-called nuclear organisation, correlates with functional status within a “healthy” nucleus and alterations in this organisation are commonly seen when nuclear function is altered or reprogrammed in disease (reviewed in: Dauer and Worman 2009; Lever and Sheer 2010; Misteli 2010; Stein et al. 2010; Rajapakse and Groudine 2011). Disease-specific patterns of chromosome territory organisation are especially notable in cancer (Cremer et al. 2003; Meaburn and Misteli 2008; Marella et al. 2009), laminopathies such as Hutchinson-Gilford Progeria syndrome (Bridger and Kill 2004; Misteli 2005; Elcock and Bridger 2010) and X-linked Emery-Dreifuss muscular dystrophy (Boyle et al. 2001).

In the context of global nuclear architecture, changes in genome organisation have been shown to

occur during differentiation in several systems (Kosak and Groudine 2004; Foster et al. 2005; Cremer and Cremer 2010). For example, gene silencing is known to be controlled by association of repressed genes with centromeric (Brown et al. 1997) or peripheral (Kosak et al. 2002) heterochromatic domains and the induction of gene expression. Gene relocation occurs towards the nuclear interior during mouse embryogenesis (Takizawa et al. 2008), neural induction (Schneider and Grosschedl 2007) and adipogenesis (Kuroda et al. 2004; Szczerbal et al. 2009). These and numerous similar observations allude to the dynamic plasticity of chromatin and demonstrate that, while interphase chromosomes clearly occupy restricted nuclear domains, the folding of chromatin within these domains has substantial flexibility (Shopland et al. 2006). Perhaps importantly, recent studies have implied that this dynamic plasticity of chromatin is sufficient to allow the formation of wide-scale networks of intra- and inter-chromosomal interaction that are now thought to play a dominant role in shaping the nuclear landscape (Fraser and Bickmore 2007; Schoenfelder et al. 2010; Rajapakse and Groudine 2011). While the range and dynamic plasticity of chromatin in mammalian nuclei requires further study, there is no doubt that the optimal “steady state” that is assumed under normal growth can be severely disrupted in disease. This study is concerned with whether the radical nuclear reorganisation that normally occurs during spermatogenesis is compromised in diseases of fertility that are known to effect gross genomic changes.

The nuclei of marsupial sperm are highly organised with a central sex chromosome, however the nuclear organisation in chicken sperm appears to be more random (Greaves et al. 2003). In higher mammals, during spermatogenesis, a highly ordered set of nuclear reorganisation events is set in place to prepare spermatozoa for fertilisation leading to a defined nuclear architecture (Haaf and Ward 1995; Hazzouri et al. 2000; Mudrak et al. 2005; Tilgen et al. 2001; Zalensky et al. 1995). Chromosome territory organisation is consequently non-random in mature sperm heads with the centromeres clustering to form the chromocentre adjacent to either the X or the Y chromosome territory at the nuclear interior (Luetjens et al. 1999; Solov'eva et al. 2004; Zalenskaya and Zalensky 2004; Zalensky et al. 1995, 1993). Similar spatial organisation is conserved in mammals as

indicated in bovine (Zalenskaya and Zalensky 2004), murine (Haaf and Ward 1995; Meyer-Ficca et al. 1998; Turner et al. 2006), porcine (Foster et al. 2005), equine and rattine (Zalenskaya and Zalensky 2004) species. In humans, the sex chromosome territories are thought to locate slightly nearer the periphery (Zalensky and Zalenskaya 2007); however, the overall picture in mammals is one with the sex chromosome territories are both peripherally located pre-meiosis, separating from one another and each repositioning to the nuclear centre by the round spermatid stage. This last stage coincides with the formation of the chromocentre (Foster et al. 2005).

Severe impairment of the spermatogenic process is extensively reported in human populations. In at least 20% of infertile couples a male factor problem is specifically implicated with genetic reasons apparently accounting for about 15% of these (Seli and Sakkas 2005). The largest single “cause” of infertility however is “idiopathic” or “unexplained” (15–20%) thereby highlighting our partial ignorance of a disorder that affects one in six couples (Ferlin et al. 2007; Seli and Sakkas 2005). The genetic (chromosomal, monogenic and multifactorial) causes of male infertility result in infertility phenotypes of various aetiologies (O’Flynn O’ Brien et al. 2010), however it seems certain that there are more still to be discovered. Numerous reports argue for associations between sperm deoxyribonucleic acid (DNA) damage and male infertility (Aitken and De Iuliis 2007; Delbes et al. 2009; Varghese et al. 2008; Zini and Libman 2006) through three major mechanisms: chromatin remodelling by topoisomerase, oxidative stress and abortive apoptosis (Aitken and De Iuliis 2009; Tarozzi et al. 2007). Aitken and De Iuliis (2009) proposed a two-step hypothesis for the origins of DNA damage in human sperm and that such damaged cells possess “hallmarks of dysmaturity” e.g. residual cytoplasm retention, compromised zona binding, inappropriate chaperone content and higher levels of nuclear histones. Delbes et al. (2009) highlighted that proper paternal genome integrity is fundamental as the sperm can potentially bring genetic damage to the resulting embryo and thereby affect the pregnancy outcome. Despite this, they point out that a direct correlation between the paternal sperm chromatin integrity and the health of the future offspring is not an easy one to make, and that further investigations are needed if this link is to be established fully. Other clinical studies however

argue that sperm DNA damage can have a detrimental effect on reproductive outcomes (including lower intrauterine insemination pregnancy rates and higher pregnancy loss following IVF/intracytoplasmic sperm injection (ICSI)) and that infertile men possess substantially more spermatozoa with DNA damage (Barratt et al. 2010; Zini and Libman 2006).

The association between increased sperm aneuploidy (i.e. a greater proportion of sperm with extra or missing chromosomes) and male infertility has been long established (Moosani et al. 1995). In a review by Tempest and Griffin (2004), results for nearly all chromosomes were summarised and there is a clear consensus for a correlation between sperm aneuploidy and male infertility, i.e. a 2–30× increase in sperm aneuploidy in males with severely compromised spermatogenesis e.g. oligoasthenoteratozoospermia—OAT (Bernardini et al. 1997; Durakbasi-Dursun et al. 2008; Gole et al. 2001; Pfeffer et al. 1999; Storeng et al. 1998; Ushijima et al. 2000; Zhang and Lu 2004). At least two studies provide evidence for the functional basis of spermatogenic defects in OAT men, namely Corrales et al. (2000), who implicated abnormal distributions of glycosidase proteins (important in fertilisation), and Liu et al. (2004), who identified greater mitochondrial dysfunction in these patients.

Given the high levels of chromosome abnormalities in the sperm of infertile men, the association with increased DNA damage, the identification of functional correlates such as mitochondrial dysfunction, the crucial role of nuclear organisation in normal spermatogenesis and the evidence of altered nuclear organisation in other diseases, the hypothesis that nuclear organisation will be altered in males with severely compromised spermatogenesis seems an obvious one to propose (Finch et al. 2008b; Ioannou and Griffin 2010; Zalensky and Zalenskaya 2007). Indeed, it is reasonable to suggest that men with measurably altered nuclear organisation in their sperm heads might have fertility problems. Convincing evidence to support this hypothesis is however still patchy at best and only a handful of studies have begun to establish a possible association (Ioannou and Griffin 2010). Luetjens et al. (1999) suggested that sperm used in intracytoplasmic sperm injection treatment that have not undergone the acrosomal reaction could impair chromatin decondensation and hinder the first cleavage division causing chromosome segregation errors. Indeed it has been postulated

that sperm with a chemically interrupted nuclear matrix (which mediates the attachment sites of compacted sperm chromatin) cannot produce viable offspring (Ward et al. 1999). Turner et al. (2006) described a mechanism for the silencing of unsynapsed chromatin associated with the formation of a chromocentre and interior sex chromosome territories. They proposed that such a mechanism might act as a control measure to prevent the gametes from excessive sperm aneuploidy. By implication if this control mechanism is impaired then increased sperm aneuploidy might ensue. Sbracia et al. (2002) reported no differences in the longitudinal position of the sex chromosomes between normal and oligozoospermic males undergoing ICSI treatment. Similarly, Olszewska et al. (2008) compared longitudinal positions for chromosomes 15, 18, X and Y between control males and infertile patients without finding a difference in nuclear organisation. In contrast, Wiland et al. (2008) found inter-individual differences in centromere topology between normal males and reciprocal translocation carriers. Thus far, the only study of which we are aware that examined the radial position for structures expected to reside at the nuclear centre (centromeres of X and 18 and the long arm of the Y) in fertile and infertile males are that of our own laboratory (Finch et al. 2008b). We suggested that all three loci occupied interior positions in normal males, but that the sex chromosomes showed altered positions (a more random distribution) in some of the infertile patients. In the current study, we extend these investigations to all autosomes for which peri-centromeric probes are available (chromosomes 1–4, 6–12, 15–18, 20, X and Y). We test the hypothesis that the nuclear organisation of the chromocentre and the sex chromosomes is significantly altered in men with severely compromised spermatogenesis as manifested by poor semen parameters (OAT) and high levels of sperm aneuploidy. We reason that, if this hypothesis is correct, then it could form the basis for the identification of, and screening for, particular types of male infertility.

## Materials and methods

### Sperm samples

Informed, written consent was obtained from 10 chromosomally normal donor males (control group)

and from 10 chromosomally normal males undergoing ICSI treatment for male factor infertility (test group). Samples were obtained from the Embryogenesis Clinic in Athens, Greece and from the London Bridge Fertility, Gynaecology and Genetics Centre, UK. Research was approved by the Research Ethics Committees of the University of Kent and carried out under the auspices of the treatment licence awarded by the Human Fertilisation and Embryology Authority and Hellenic National Authority of Assisted Reproduction (English translation of Greek acronym EAITA) to the London Bridge and Embryogenesis clinics, respectively. Table 1 shows the patient information concerning all males in the study and semen parameters were determined using strict WHO criteria. All patients were non-smokers and between the ages of 20–25. In the control group, all had a sperm concentration >40 million/ml (mean 64.1 million/ml), a motility of >30% (mean 45.9%) and a sperm aneuploidy (for five chromosomes analysed) of between 0.4 and 1.1% (mean 0.67%); morphology analysis is not routinely performed on normal donors. In the test group (OAT men), all had a sperm concentration of <20 million/ml (mean 6 million/ml), a motility  $\leq$ 30% (mean 8.8%) and  $\geq$ 90% abnormal forms (mean 96.7%). Sperm aneuploidy for the five chromosomes analysed ranged between 0.4% and 4.0% (mean 2.42%). Thus the control group collectively showed evidence of relatively normal spermatogenesis whereas, in the test group, spermatogenesis was severely impaired.

### Sperm sample preparation

Ejaculated sperm was washed in 10 mM NaCl/10 mM Tris pH 7.0 sperm wash buffer and then centrifuged for 7 min at 700 $\times$ g. The supernatant was removed and resuspended in wash buffer three to five times depending on the sample quality (pellet size and colour). The sample was then fixed in a drop-wise fashion using 3:1 methanol acetic acid fixative to a final volume of 5 ml. Again it was centrifuged at 700 $\times$ g for 7 min and, after removal of the supernatant, the pellet was resuspended in the same fixative. The process was repeated for up to five times (pellet depending). Between 5 and 20  $\mu$ l of the sample was spread on a poly-L-lysine-coated slide (allowing superior adherence of sperm cells—631-0107-VWR; West Chester, PA, USA) and allowed to air dry at room temperature. The optimal density of cells was

**Table 1** Semen parameters and % sperm disomy (for five chromosome pairs) for the 10 control males (N1–N10) and the 10 OAT “test” males (OAT1–OAT10)

| Sample       | Age  | Concentration (10 <sup>6</sup> /ml) | Motility (%) | Progressive motility (%) | Abnormal forms (%) | % sperm disomy (five chromosomes) |
|--------------|------|-------------------------------------|--------------|--------------------------|--------------------|-----------------------------------|
| N1           | 37   | 60                                  | 35           | — <sup>a</sup>           | — <sup>a</sup>     | 0.7                               |
| N2           | 27   | 52                                  | 33           | — <sup>a</sup>           | — <sup>a</sup>     | 0.4                               |
| N3           | 33   | 52                                  | 33           | — <sup>a</sup>           | — <sup>a</sup>     | 0.7                               |
| N4           | 44   | 107                                 | 82           | — <sup>a</sup>           | — <sup>a</sup>     | 0.6                               |
| N5           | 41   | 45                                  | 39           | — <sup>a</sup>           | — <sup>a</sup>     | 0.5                               |
| N6           | 38   | 54                                  | 41           | — <sup>a</sup>           | — <sup>a</sup>     | 0.9                               |
| N7           | 25   | 49                                  | 44           | — <sup>a</sup>           | — <sup>a</sup>     | 0.6                               |
| N8           | 23   | 79                                  | 31           | — <sup>a</sup>           | — <sup>a</sup>     | 1.1                               |
| N9           | 35   | 100                                 | 90           | — <sup>a</sup>           | — <sup>a</sup>     | 0.6                               |
| N10          | 22   | 43                                  | 31           | — <sup>a</sup>           | — <sup>a</sup>     | 0.6                               |
| Mean control | 32.5 | 64.1                                | 45.9         | — <sup>a</sup>           | — <sup>a</sup>     | 0.67                              |
| OAT1         | 40   | 18                                  | 30           | 20                       | 90                 | 3.3                               |
| OAT2         | 29   | 10                                  | 20           | 10                       | 95                 | 2.6                               |
| OAT3         | 36   | 8                                   | 10           | 5                        | 97                 | 4.1                               |
| OAT4         | 41   | 6                                   | 5            | 1                        | 98                 | 1.6                               |
| OAT5         | 37   | 5                                   | 5            | 2                        | 98                 | 4.0                               |
| OAT6         | 50   | 5                                   | 5            | 1                        | 96                 | 3.9                               |
| OAT7         | 40   | 2                                   | 1            | 0                        | 98                 | 1.6                               |
| OAT8         | 34   | 1                                   | 5            | 2                        | 100                | 1.0                               |
| OAT9         | 52   | 3                                   | 5            | 2                        | 99                 | 1.7                               |
| OAT10        | 48   | 2                                   | 2            | 1                        | 96                 | 0.4                               |
| Mean test    | 40.7 | 6.0                                 | 8.8          | 4.4                      | 96.7               | 2.42                              |

<sup>a</sup> Progressive motility and morphology are not routinely tested for normal donors at the clinics from which we received our samples

then ascertained with the aid of a phase contrast microscope and areas of interest marked with a diamond pen.

#### Sperm fluorescence in situ hybridisation

Slides were aged for 1 h at 70°C on a hot block then dehydrated in an ethanol series (70–80–100% for 3 min each). Sperm cells were then incubated in 10 mM DTT (Sigma-Aldrich, St Louis, MO, USA) 0.1 M Tris–HCl (pH 8.0) for 20–30 min at room temperature (in the dark) to swell them, and then rinsed in 2× saline sodium citrate (SSC). A 0.01% pepsin treatment (in 0.01 M HCl, Fisher, Pittsburg, PA, USA) for 20 min, preceded rinses in double-distilled water (ddH<sub>2</sub>O) and phosphate-buffered saline (PBS). A further fixation in 1% paraformaldehyde/PBS continued at 4°C for 10 min. Slides were then rinsed with PBS then ddH<sub>2</sub>O at room temperature, then dehydrated in an ethanol series and air-dried.

Fluorescently labelled in situ hybridisation (FISH) probes (Kreatech Diagnostics, Amsterdam,

the Netherlands—see Table 2) were denatured at 72°C for 10 min, then added to the slide before sealing the region of interest with Parafilm<sup>®</sup>. This was followed by a co-denaturation with the target cells (sperm) at 75°C for 90 s using a Thermobrite<sup>®</sup> Statspin (Abbott Molecular, Illinois, IL, USA). This was followed by hybridisation at 37°C for 15 min. Slides were removed from the Thermobrite<sup>®</sup>, and the Parafilm<sup>®</sup> was carefully removed. Post-hybridisation stringency washes continued in 0.7× SSC–0.3% Tween 20 (Sigma-Aldrich, St Louis, MO, USA), at room temperature, to allow the coverslips to float off, then at 72°C in the same solution for 3 min. Slides were then transferred to 2× SSC at room temperature for 2 min before briefly washing in ddH<sub>2</sub>O and staining with 0.1 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS. Slides were then mounted in Vectashield (Vector labs, Burlingame, CA, USA) under a coverslip. Analysis proceeded on an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera (Hamamatsu Orca-ER C4742-80) using bespoke single band pass

**Table 2** Locus specific probes used in this study

| Fluorochrome     | Probe combination 1           | Probe combination 2           | Probe combination 3      |
|------------------|-------------------------------|-------------------------------|--------------------------|
| PB405 dark blue  | Chromosome 7 centromere       | Chromosome 11 centromere      | Chromosome 18 centromere |
| PB415 light blue | Chromosome 1 peri-centromeric | Chromosome 9 peri-centromeric | Chromosome 16 centromere |
| PB495 green      | Chromosome 6 centromere       | Chromosome 20 centromere      | Chromosome 2 centromere  |
| PB547 orange     | Chromosome 8 centromere       | Chromosome 12 centromere      | X chromosome centromere  |
| PB590 red        | Chromosome 3 centromere       | Chromosome 10 centromere      | Y chromosome centromere  |
| PB647 far-red    | Chromosome 4 centromere       | Chromosome 17 centromere      | Chromosome 15 centromere |

Each probe combination is listed along with the fluorochrome used to label it

*PB* platinum bright

filters for each fluorochrome (Chroma Technology, Bellows Falls, VT, USA). All images were acquired using SmartCapture software (Digital Scientific UK) and exported as tiff files for further analysis.

### Probes

The probes used in this study are summarised in Table 2. Each mix consisted of repetitive (e.g. centromeric) sequences targeting six chromosomes each labelled with a different fluorochrome. All probes were provided by Kreatech Diagnostics (Amsterdam, The Netherlands). Probes were available only for chromosomes 1–4, 6–12, 15–18, 20, X and Y as those for the remaining chromosomes are not chromosome-specific as they cross react with one another (13 and 21, 14 and 22 and 5 and 19).

### Analysis of nuclear organisation

In each male, for each probe, the question was asked whether a non-random pattern of distribution could be identified after analysis of 100 signals. If so, which part of the nucleus was preferentially occupied with reference to five “shells,” each representing equal portions of the sperm head (from periphery to interior). This approach was first established by Croft et al. (1999); we used a “macro” designed in-house and written in ImageJ (Skinner et al. 2009) that encapsulated the principles established by Croft et al. (1999) and Boyle et al. (2001). Briefly, this macro first split each image of a nucleus into separate RGB planes (red and green for two of the six signals, blue for the DAPI counterstain) and then converted the blue image (representing the DAPI counterstain) to a binary mask from which concentric regions of interest

(rings) of equal area were created. The proportion of signal in each channel contained within each shell was measured relative to the total signal for that channel within the area covered by the binary mask (Skinner et al. 2009). The numerical output of these results was copied and pasted to an excel spreadsheet for statistical analysis. To compensate for the fact that we were essentially deriving three-dimensional (3D) information from a flattened two-dimensional (2D) object the proportion of signal within each shell was normalised against the DAPI density measured within that shell as a function of the amount of DNA measured (Boyle et al. 2001). This is a standard approach used in the majority of studies of this kind. Thus signals in shell 1 (the peripheral shell) received proportionally higher “scores” than those in the interior shell (shell 5) to adjust for the fact that centrally located observations were statistically more likely in a random distribution. The results were represented as a histogram and a  $\chi^2$  test was performed to test whether the nuclear position of the signal was non-randomly distributed to a specific shell ( $p < 0.05$ ) or “not discernible from a random pattern” (NDRP) after analysis of 100 cells. The corrected value of the overall positions was taken for all nuclei scored for a specific probe (i.e. the highest bar on the histogram) and taken to be the preferential nuclear address for that probe.

Thus, for all the data presented in this study, the following criteria were used (based on the highest bar (s) in the histogram) in order to classify the relative preferential nuclear address for each locus analysed:

- Shell 1 or 1–2=“Peripheral”
- Shell 2 or 1–3=“Peripheral/medial”
- Shell 3, 2–3 or 3–4=“Medial”

- Shell 4 or 3–5=“Interior/medial”
- Shell 5 or 4–5=“Interior”
- “Bimodal” = Highest bars in two non-adjacent shells, e.g. 2 and 4
- “NDRP” = not discernible from a random pattern after analysis of 100 cells

## Results

### Chromosome loci position in control and test groups

In total 19,293 signals were analysed for 18 (peri-)centromeric loci in 10 different men with normal semen parameters (control group). On average 107 data points (signals) were recorded and analysed per locus, per male. With regard to men with OAT (test group), 18,752 signals for all loci were analysed from 10 different men. On average for each probe, for each male, 104 images were analysed. Figure 1 is an example of a typical result from a FISH experiment.

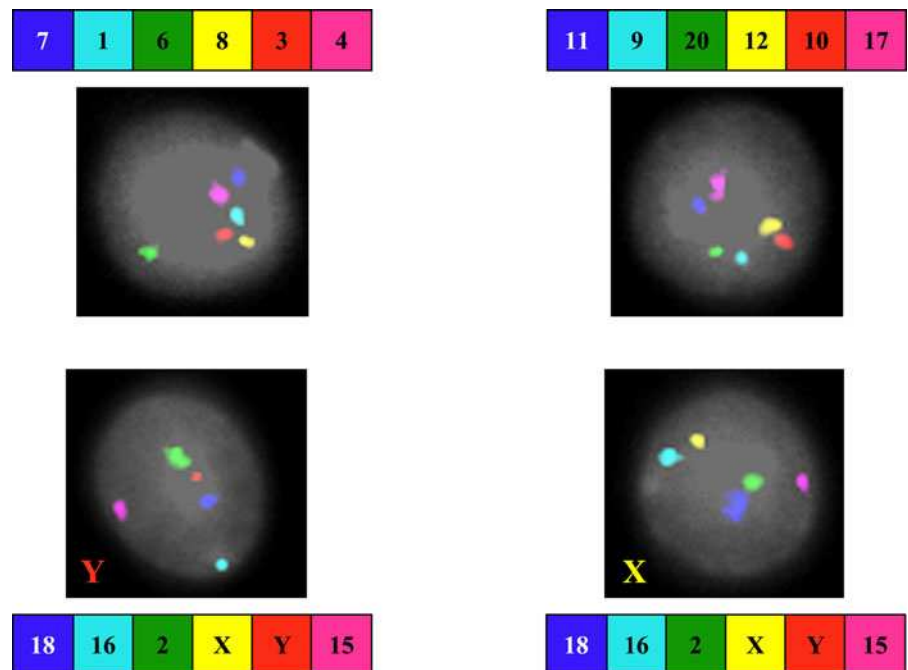
The results for the preferential nuclear address for each locus, for each male are summarised in Tables 3 and 4. A summary of the pooled results for the sex chromosomes both test (right) and control (left) groups is shown in Fig. 2 with the full dataset in the

supplementary figures I–VI. Evidence for a chromocentre in the sperm heads of normal males is clearly demonstrated as, in each individual control male, all chromosomal loci positions were non-randomly distributed ( $p < 0.05$  by chi-squared test). Each individual nuclear address could be described as either “interior” or “interior/medial” with rare “medial” exceptions in selected males (seven in total) for chromosomes 3, 6, 8 and 18.

A chromocentre was also apparent in most males in the test group; however there were subtle differences from the controls. That is, over twice as many signal distributions could be described as “medial” (16 in total, for chromosomes 3, 6, 12 and 18) with four analyses falling into the “NDRP” category (for chromosomes 4, 6 and 12).

When considering the “pooled” data (i.e. all males in the test and control groups pooled together per chromosome, Fig. 2 and supplementary figures I–VI), further subtle differences were apparent. That is, for all loci examined in the control group, pooled data indicated an “interior” or rarely an “interior/medial” position for all chromosomes, providing further evidence for the presence of a chromocentre and interior sex chromosome territories. In the test group however, the centromeres of chromosomes 3 and 6 clearly adopted a “medial” position overall, with the

**Fig. 1** Example of sperm FISH results using the multicolour probe sets used in this study





**Table 3** The nuclear address for all the loci analysed in each of the control males

| Locus | Shell 3<br>“medial” | Shell 3–4<br>“medial” | Shell 3–5<br>“interior–medial” | Shell 4<br>“interior–medial” | Shell 4–5<br>“interior” | Shell 5<br>“interior” | NDRP |
|-------|---------------------|-----------------------|--------------------------------|------------------------------|-------------------------|-----------------------|------|
| X     | 0                   | 0                     | 0                              | 2                            | 3                       | 5                     | 0    |
| Y     | 0                   | 0                     | 0                              | 1                            | 1                       | 8                     | 0    |
| 1     | 0                   | 0                     | 1                              | 0                            | 4                       | 5                     | 0    |
| 2     | 0                   | 0                     | 2                              | 3                            | 1                       | 4                     | 0    |
| 3     | 0                   | 1                     | 3                              | 1                            | 2                       | 3                     | 0    |
| 4     | 0                   | 0                     | 2                              | 0                            | 3                       | 5                     | 0    |
| 6     | 1                   | 2                     | 2                              | 0                            | 4                       | 1                     | 0    |
| 7     | 0                   | 0                     | 0                              | 0                            | 6                       | 4                     | 0    |
| 8     | 2                   | 0                     | 1                              | 1                            | 5                       | 1                     | 0    |
| 9     | 0                   | 0                     | 0                              | 3                            | 1                       | 6                     | 0    |
| 10    | 0                   | 0                     | 0                              | 2                            | 4                       | 4                     | 0    |
| 11    | 0                   | 0                     | 1                              | 1                            | 1                       | 7                     | 0    |
| 12    | 0                   | 0                     | 1                              | 1                            | 4                       | 4                     | 0    |
| 15    | 0                   | 0                     | 0                              | 0                            | 1                       | 9                     | 0    |
| 16    | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| 17    | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| 18    | 0                   | 1                     | 1                              | 1                            | 2                       | 5                     | 0    |
| 20    | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| Total | 3                   | 4                     | 14                             | 16                           | 51                      | 92                    | 0    |

In each cell of the table, the numbers represent the number of men in which a certain pattern was seen for each chromosome. There were no cases in which a pattern of distribution for a particular locus in a particular male was seen in shells 1 or 2 (the outermost shells) nor a pattern not discernible from random (NDRP)

centromeres of chromosomes 12 and 18 adopting an “interior/medial” position compared to the “interior” position in the controls.

Taken together the results suggest that certain loci are more prone to a random distribution or subtle repositioning. The centromeres for chromosomes 3, 4, 6, 8, 12 and 18 seem to be the ones that are usually involved. There is not however a complete breakdown to the extent that all of the loci appear randomly distributed. In other words we cannot find evidence of a radically different nuclear organisation associated with severely compromised spermatogenesis.

## Discussion

In the current study we provide further evidence that centromeric and sex chromosome loci adopt specific nuclear addresses towards the interior of the nucleus, consistent with interior sex chromosome territories and a chromocentre (Zalenskaya and Zalensky 2004; Zalensky et al. 1995). Moreover, limited alteration in the nuclear positioning of the chromocentre is associated with compromised spermatogenesis. Given the degree of sperm aneuploidy and/or the levels of

DNA damage associated with male infertility however, it is perhaps somewhat surprising that we did not see a greater degree of difference between test and control groups. In future studies we will consider assays to determine DNA damage e.g. comet assay (Tarozzi et al. 2007) as it would be interesting to investigate whether, for instance, the men who showed apparently random patterns were also those with the highest levels of DNA damage.

During spermiogenesis histones are replaced first by transition proteins (Meistrich et al. 2003), then by protamines (Balhorn 1982) with only 15% of chromatin remaining bound to the histones (Wykes and Krawetz 2003). The resulting increase in arginine (Bjorndahl and Kvist 2009) means that  $-\text{NH}_3^+$  groups neutralise the negative charges of the phosphate groups in the DNA backbone allowing a higher degree of compaction (Bjorndahl and Kvist 2009). This compaction is clearly accompanied by a radical alteration of the orientation of the chromosome territories and repositioning of the sex chromosomes and centromeres to the nuclear centre. However, according to the results of this study, this process occurs largely unaltered, despite considerable dysfunction upstream in the spermatogenic process. In some ways this is

**Table 4** The nuclear address for all the loci analysed in each of the OAT males

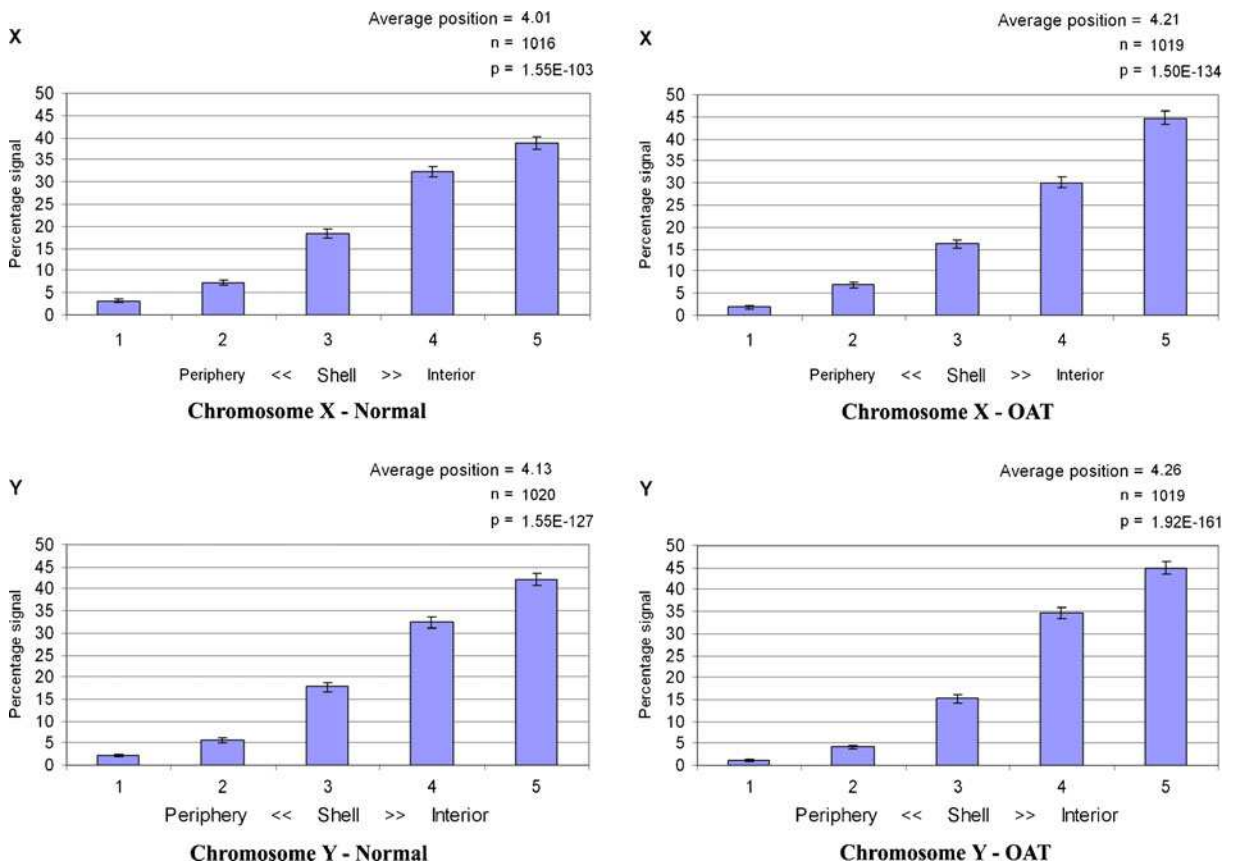
| Locus | Shell 2–4<br>“bimodal” | Shell 3<br>“medial” | Shell 3–4<br>“medial” | Shell 3–5<br>“interior–medial” | Shell 4<br>“interior–medial” | Shell 4–5<br>“interior” | Shell 5<br>“interior” | NDRP |
|-------|------------------------|---------------------|-----------------------|--------------------------------|------------------------------|-------------------------|-----------------------|------|
| X     | 0                      | 0                   | 0                     | 0                              | 0                            | 1                       | 9                     | 0    |
| Y     | 0                      | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| 1     | 0                      | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| 2     | 0                      | 0                   | 0                     | 2                              | 2                            | 3                       | 3                     | 0    |
| 3     | 0                      | 4                   | 0                     | 3                              | 2                            | 0                       | 1                     | 0    |
| 4     | 1                      | 0                   | 3                     | 2                              | 0                            | 1                       | 1                     | 2    |
| 6     | 0                      | 3                   | 2                     | 2                              | 0                            | 2                       | 0                     | 1    |
| 7     | 0                      | 0                   | 0                     | 2                              | 1                            | 4                       | 3                     | 0    |
| 8     | 0                      | 0                   | 0                     | 3                              | 2                            | 0                       | 5                     | 0    |
| 9     | 0                      | 0                   | 0                     | 1                              | 2                            | 3                       | 4                     | 0    |
| 10    | 0                      | 0                   | 0                     | 0                              | 1                            | 5                       | 4                     | 0    |
| 11    | 0                      | 0                   | 0                     | 2                              | 0                            | 3                       | 5                     | 0    |
| 12    | 0                      | 1                   | 1                     | 2                              | 2                            | 3                       | 0                     | 1    |
| 15    | 0                      | 0                   | 0                     | 0                              | 1                            | 2                       | 7                     | 0    |
| 16    | 0                      | 0                   | 0                     | 0                              | 1                            | 1                       | 8                     | 0    |
| 17    | 0                      | 0                   | 0                     | 0                              | 1                            | 1                       | 8                     | 0    |
| 18    | 0                      | 0                   | 1                     | 2                              | 4                            | 2                       | 1                     | 0    |
| 20    | 0                      | 0                   | 0                     | 0                              | 0                            | 3                       | 7                     | 0    |
| Total | 1                      | 8                   | 7                     | 21                             | 19                           | 40                      | 80                    | 4    |

In each cell of the table, the numbers represent the number of men in which a certain pattern was seen for each chromosome. There were no cases in which a pattern of distribution for a particular locus in a particular male was seen in shell 1 (the outermost shell) or shell 2 alone

surprising because the process of reorganisation presumably requires energy and it has been suggested that mitochondrial dysfunction is a hallmark of severe OAT (Liu et al. 2004). Indeed if repositioning of the centromeres and sex chromosomes were driven by transcriptional activity (Olszewska et al. 2008), upstream transcriptional alterations in infertile men should, theoretically, result in marked differences in nuclear organisation. The reasons for the association between the tight packing of the nucleus mediated by the protamines and the reorganisation of the chromosome territories therefore warrants further investigation and it has been suggested that efficient packaging is essential to facilitate proper delivery of the paternal genome to the egg (reviewed in Miller et al. 2010). Ward (2009) however argued that the cysteine residues of protamines confer extra stability in the sperm chromatin through intermolecular disulphide cross-linking and therefore sperm chromatin rearrangement functions to ensure proper fertilisation as a protective agent of the paternal genome, not for future embryonic

development. Studies of early cleavage divisions in human embryos (e.g. Finch et al. 2008a) might shed light on this in future.

The reasons for the subtle alterations being restricted to particular centromeres such as those for chromosomes 3, 4, 6, 8, 12 and 18 are not clear, however, they may point to a chromosome-specific “hierarchy” of centromeric positions within the chromocentre. Our data provides preliminary evidence for this in that, for certain chromosomes, the overall distribution appears different (e.g. relatively higher bars in shell 5). Evidence suggests that extrapolations from 2D data (such as that presented in this study) are an indicator of real 3D positions (Federico et al. 2008); however, this data relates to lymphocyte nuclei and it is possible that sample preparation can affect nuclear organisation. It would therefore perhaps be unwise to over-interpret these 2D projections and further experiments on preserved 3D sections, perhaps coupled with confocal microscopy and/or deconvolution algorithms could be used to



**Fig. 2** Pooled distribution of nuclear position for the sex chromosome centromeres in 10 normal (*left*) and in 10 OAT (*right*) males

establish whether e.g. chromosomes 6 and 8 do not occupy quite such a central position as some of the others. Further similar experiments could also be carried out involving other chromosomal structures such as the telomeres to test the hypothesis that they occupy peripheral positions as previously proposed (Zalenskaya and Zalensky 2004; Zalensky et al. 1995) and whether this is perturbed in men with severely compromised spermatogenesis.

The only directly comparable study of which we are aware that has assessed the radial positions of chromosome loci in normal and infertile men is that of our own laboratory (Finch et al. 2008b). Men with a range of fertility phenotypes were examined for three loci (on chromosomes X, Y and 18) and we reported that the centromere of chromosome 18 remained resolutely interior, regardless of the phenotype. In the current study we largely confirm these results, but with a greater number of centromeres (albeit chromosome 18 being predominantly occupying shell 4 in this study for OAT males). We also

however previously reported topological alterations of the positions of the sex chromosomes associated with increased severity of infertility, an observation not supported by the current study where only OAT males were examined. It might be argued that a general picture of a small but significant breakdown of the nuclear organisation of sperm heads associated with infertility is emerging, however this notion needs investigating on a much larger series of men before it can be confirmed or refuted.

Zalensky and Zalenskaya (2007) propose that in addition to concentration, motility, morphology, DNA damage and aneuploidy, a new class of sperm chromosome abnormality related to atypical packing of chromosome territories and/or aberrant nuclear position should be considered. This could have an impact on fertilisation and early embryogenesis (Finch et al. 2008a). Based on the results of the current study, the loci for the centromeres of chromosomes 3, 4, 6 and 12 might be a good starting point. However, differences are subtle and it remains to be seen whether, in

practice, a chromosome position assay would realistically find its way into routine clinical practice.

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