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# Non-random chromosome positioning in mammalian sperm nuclei, with migration of the sex chromosomes during late spermatogenesis

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## **Summary**

Chromosomes highly organized compartmentalized in cell nuclei. The analysis of their position is a powerful way to monitor genome organization in different cell types and states. Evidence suggests that the organization of the genome could be functionally important for influencing different cellular and developmental processes, particularly at early stages of development (i.e. fertilization and the consequent entry of the sperm nucleus into the egg). The position of chromosomes in the sperm nucleus might be crucial, because their location could determine the time at which particular chromatin domains are decondensed and remodelled, allowing some epigenetic level of control or influence over subsequent paternal gene expression in the embryo. Here, we analyse genome organization by chromosome position in mammalian sperm nuclei from three breeds of pig, as a model species. We have mapped the preferential position of all chromosomes (bar one) in sperm nuclei in two dimensions

and have established that the sex chromosomes are the most internally localized chromosomes in mature sperm. The distribution of two autosomes and chromosomes X and Y in sperm heads was compared in primary and secondary spermatocytes and spermatids in porcine testes. The sex chromosomes were found at the nuclear edge in primary spermatocytes, which correlates with the known position of the XY body and their position in somatic cells, whereas, in spermatids, the sex chromosomes were much more centrally located, mirroring the position of these chromosomes in ejaculated spermatozoa. This study reveals the temporal repositioning of chromosome territories in spermatogenesis.

Key words: Chromosome position, Sperm nuclei, Porcine development, Genome organization, Nuclear organization, Spermatogenesis

## Introduction

By studying the spatial order and organization of chromosomes in interphase nuclei, we are beginning to understand much more about the genome, mainly about higher levels of control and regulation that go beyond the influence of sequence alone. From such studies, it has been demonstrated that, within interphase nuclei, chromosomes are highly organized and compartmentalized into their own specific regions, known as chromosome territories (Cremer et al., 1988; Lichter et al., 1988). Indeed, chromosome arm domains and specific chromosomal bands also occupy their own distinct region of the nucleus without intermingling with other closely associated domains (Dietzel et al., 1998a; Dietzel et al., 1998b; Sadoni et al., 1999).

By using analyses that determine interphase chromosome position, a correlation has been demonstrated between the positioning of chromosome territories in interphase nuclei of proliferating cells and the gene density of the chromosome (Croft et al., 1999; Boyle et al., 2001). That is, human chromosomes have been found to occupy reproducibly specific

nuclear addresses, with an internal nuclear positioning for chromosomes with a high gene density and a more peripheral nuclear position for chromosomes with a low gene density. This organization of gene-dense and less-gene-dense chromosomes is evolutionarily conserved, because primates and Old-World monkeys display a similar positioning for chromosomes or chromosome regions syntenic to human chromosomes (Tanabe et al., 2002). Furthermore, in the chicken (Gallus domesticus), chromosome positioning has also been investigated in fibroblasts and neurons, and exhibits similar radial chromosome positioning to human and primates with gene-rich microchromosomes towards the nuclear interior and gene-poor macrochromosomes at the edge of the nucleus (Habermann et al., 2001). However, this positioning also fits an alternative hypothesis, whereby chromosomes are positioned according to their size, because the more-gene-rich chromosomes are also the small microchromosomes (McQueen et al., 1996; McQueen et al., 1998; Smith et al.,

Making cells quiescent or senescent results in altered spatial

positioning of chromosomes HSA18 and HSA13 (Bridger et al., 2000) (K. J. Meaburn and J.M.B., unpublished) and is an example of when a change in cell status affects genome organization, which might in turn influence the role and behaviour of the genome. A second example in which chromosome positioning is altered is in some cancer cells, in which the positioning of internal chromosomes is altered (Cremer et al., 2003). Indeed, in HL-60 cells induced to differentiate by dimethyl sulfoxide, specific genetic loci were repositioned in the differentiated cells (Skalnikova et al., 2000). It has also been shown that regions of high gene density loop out of chromosome territories when they are actively transcribing (Mahy et al., 2002; Williams et al., 2002; Volpi et al., 2000). Thus, it has been clearly demonstrated that there can be dynamic alterations to genome organization in cells regulated by different signalling pathways, whether normal or aberrant.

In this study, we have concentrated on analysing the genome organization in a highly specialized cell type, spermatozoa. This is partly to provide the ground work for studies to understand the role of the genome in gametogenesis, fertilization, fertility and gene expression patterns in the early developing embryo. We hypothesize that chromosome and locus positioning is crucial in the control and modification of the genome, especially when the paternal genome comes into contact with oocyte cytoplasm and that chromosome positioning in sperm is very different to other cells. To test this hypothesis, we have analysed the position of chromosomes in mature sperm and in sperm precursor cells in spermatogenesis.

Only a few investigations of genome organization have been published about sperm nuclei. Chromatin organization in spermatozoa differs vastly from that of somatic cells. Indeed, in pigs and other species, the integral structure of chromatin differs in sperm nuclei, because DNA is associated with small basic nuclear proteins known as protamines instead of with histones as in other cell types (Tobita et al., 1982; Dadoune, 1995). Spermatozoa apparently also lack gene expression, DNA repair and DNA replication (Ward, 1994; Ward and Zalensky, 1996; Hazzouri et al., 2000), but have developed from cells that were once active in these processes, making the sperm nucleus a unique and fascinating structure to analyse with respect to genome organization and control. There are, however, some studies that imply that active transcription is apparent in sperm nuclei (for a review, see Kramer and Krawetz, 1997).

Evidence from the literature shows us that chromosomes in sperm nuclei are also organized as distinct territories and that there is non-random chromosome positioning in several different species. Territories of rat chromosomes 2 and 12 are preferentially distributed within the median and tip and median regions, respectively, in both elongated spermatids and epididymal sperm (Meyer-Ficca et al., 1998). Rat sperm heads also demonstrated a non-random positioning of telomeres (Meyer-Ficca et al., 1998), although positioning of telomeres appears to be species specific and varies according to the concentrations of dithiothreitol (DTT) or heparin to which the samples were exposed in order to decondense the genome (Zalensky et al., 1997). As for human sperm, some longitudinal chromosome positioning has been revealed; for example, the positioning of chromosomes X and 7 appear to be highly organized, with a preferential location in the anterior region of the sperm (Luetjens et al., 1999; Hazzouri et al., 2000; Zalenskaya and Zalensky, 2004), chromosome 1 is less anterior but still towards the apex of the sperm (Zalenskaya and Zalensky, 2004), and chromosomes 18 and 6 are located towards the posterior of the sperm (Luetjens et al., 1999; Zalenskaya and Zalensky, 2004). There is also some evidence for chromosomes being organised radially in sperm nuclei. Zalenskaya and Zalensky (Zalenskaya and Zalensky, 2004) show that, in humans, HSA7 and HSA6 are mostly peripheral in location, with HSA16 and HSAX being more internal. These data support the observation that, in humans, the X chromosome would be one of the first chromosomes to enter the egg. Conversely, chromosome 13 appears to have a random distribution in human sperm (Hazzouri et al., 2000). Nonrandom longitudinal chromosome organization within sperm nuclei has also been demonstrated in evolutionarily divergent animals such as marsupial and monotreme mammals (Watson et al., 1996; Greaves et al., 2001; Greaves et al., 2003). The two monotremes Platypus and Echidna have long fibrillar sperm heads that display an anterior apical localization of the X chromosome. In the wombat and dunnart (both marsupials), the X chromosomes were found not at the anterior end but more in the central region. However, this is still the region of the sperm that touches the egg first (Greaves et al., 2003). Thus, it appears that marsupials display central sex (X) chromosome nuclear positioning, whereas other eutherians (including human and monotremes) have chromosome X near the edge, at the apex of the sperm nucleus. Taken together, all these data indicate that longitudinal and/or radial organization of chromosomes in sperm heads, in various animals, must be functionally significant. By contrast, two studies have provided evidence that the arrangement of chicken chromosomes within sperm nuclei is random. This was demonstrated with the use of telomeric, repetitive and whole macrochromosome paints (Solovei et al., 1998; Greaves et al., 2003). However, nonrandom radial positioning was tentatively observed, with a tendency for microchromosomes to be positioned within the interior of sperm nuclei (Greaves et al., 2003). Given the available evidence, the existence of non-random longitudinal chromosome organization within sperm nuclei seems to be a facet of the divergence of birds and mammals.

Very little, however, can be gleaned from the literature on comparisons of chromosome positioning in sperm to somatic cells. This is because many of the chromosome position assignments in sperm are examined longitudinally and very few chromosomes have been analysed. However, in a 2004 study, centromeric regions of chromosomes were positioned radially in sperm heads (Zalenskaya and Zalensky, 2004). Two chromosomes (HSA7 and HSA16) were positioned similarly to how they would be in somatic cells (Boyle et al., 2001) and two chromosomes were positioned differentially (HSAX and HSA6). To answer the question of why genome positioning in sperm differs so much from other somatic cell types, we use the pig to make comparisons of nuclear positions of all chromosomes between many cell types, developmental stages and cell states. In this study, we analyse chromosome positioning in pig sperm and in precursor cells in spermatogenesis.

By studying genome organization and chromosome positioning within porcine spermatozoa, we can gain a unique insight into nuclear organization in gametes and its implications for early development, fertility and paternal gene expression, and also into behavioural characteristics of the genome with respect to evolution. Our data show that chromosome territories are present in pig sperm and are nonrandomly distributed both longitudinally and radially. The position of the sex chromosomes differs slightly from other mammalian species, because they are centrally located. Interestingly, by analysing the positions of the sex chromosomes in nuclei of spermatocytes and spermatids in pig testis, we reveal the timing and cell stage in spermatogenesis at which the sex chromosomes change their nuclear position from a peripheral position to an internal one.

#### **Materials and Methods**

# Spermatozoa preparation

Ejaculated sperm was collected from boars housed in Sygen International's farms. The sperm-rich fraction (isolated using an IVM Accucell) of the ejaculate was incubated with sperm-wash buffer (0.01 M Tris-HCl and 0.01 M NaCl, pH8.0) for transportation. Semen samples were centrifuged for 5 minutes at 400 g before resuspending in fresh sperm-wash buffer. This was repeated twice, with a final resuspension in 1 ml sperm-wash buffer. 10  $\mu$ l resuspended sample was applied to each slide. After air drying, the samples were fixed for 2 hours at room temperature in 3:1 methanol:acetic-acid. The samples were allowed to air dry before taking them through an ethanol series of 70%, 80% and 100% for 5 minutes each. Slides were stored at  $-20^{\circ}\text{C}$  in an airtight box containing silica until use.

#### Spermatozoa decondensation

The slides were left at room temperature overnight to age them. Decondensation of spermatozoa was performed by incubating the samples with 0.01 M DTT in 0.1 M Tris, pH 8.0, for 30 minutes at room temperature and in darkness. After swelling, the samples were rinsed briefly in  $2\times$  SSC, pH 7.0 ( $1\times$  SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and allowed to air dry before being dehydrated in a 70%, 80% and 100% ethanol series.

### Tissue-section preparation

Porcine testes were incubated with sterile 40% sucrose for 3 days. The testes were cut into small pieces, frozen in a hexane bath and stored at -80°C. 60 µm sections of frozen testis tissue were cut using a cryomicrotome (Bright 5030 microtome) and adhered to slides coated with 3% 3-aminopropyltriethoxysilane (APES). The tissue sections were stored at -80°C until use. At all stages throughout the tissuesection preparation, care was taken that the samples did not air dry. This preserved the three-dimensional conformation of nuclei within the testis tissue. The tissue sections were fixed with 4% paraformaldehyde in PBS at room temperature for 10 minutes. The sections were washed in PBS and permeabilized in 0.5% saponin (w/v) and 0.5% Triton X-100 (v/v) in PBS for 25 minutes. They were then rinsed in PBS and incubated in 0.1 N HCl for 10 minutes before being rinsed again in PBS. The tissue sections were digested with 200 μg ml<sup>-1</sup> RNase A at 37°C for 1 hour. These were then washed and stored in PBS until denaturation.

#### Probe preparation

Whole porcine chromosomes were isolated by flow sorting of chromosomes prepared from peripheral blood lymphocytes (M. A. Ferguson-Smith, Molecular Cytogenetics Laboratory, Cambridge, UK). The chromosome templates underwent primary and secondary amplification by performing degenerate-oligonucleotide-primed PCR (DOP-PCR) and were subsequently labelled with biotin-16-dUTP

(Boehringer Mannheim) and digoxigenin-11-dUTP (Boehringer Mannheim) (Telenius et al., 1992). 300 ng biotin-16-dUTP-labelled chromosome paint, 50  $\mu g$  sheared porcine genomic DNA and 3  $\mu g$  herring sperm were ethanol precipitated at  $-80^{\circ}C$  for at least 1 hour before dissolving in hybridization mixture (50% formamide, 10% dextran sulphate, 2× SSC and 1% Tween 20) at 50°C for a minimum of 2 hours before performing fluorescence in situ hybridization (FISH). The probes were denatured for 5 minutes at 75°C and left for 1 hour at 37°C.

Probe preparation for 3D tissue sections of testes was identical except that 450 ng biotin-16-dUTP-labelled chromosome paint was used.

#### **FISH**

The sperm nuclei were denatured in 70% formamide,  $2 \times SSC$ , pH 7.0, at 70°C for 11 minutes before immediate immersion in ice-cold 70% ethanol for 5 minutes and passage through another ethanol series before being air-dried. The appropriate probe was applied to each slide and was covered with a 22×22 mm coverslip, sealed with rubber cement and left in a humidified chamber at 37°C overnight. On removal of the coverslips, the slides were washed three times for 5 minutes each in 50% formamide, 2× SSC, pH 7.0, at 45°C. Slides were then washed with 0.1× SSC prewarmed to 60°C but placed in a 45°C water bath, three times, for 5 minutes each, before being transferred to 4× SSC, 0.05% Tween 20 for 15 minutes at room temperature. 150  $\mu$ l 4 $\times$  SSC, 0.05% Tween 20 and 3% bovine serum albumin (BSA) blocking solution was applied to the slide and incubated for 20 minutes at room temperature. The excess was removed and a mixture containing 120 µg ml<sup>-1</sup> streptavidin Cy3 (Amersham), 200 µg antidigoxigenin-fluorescein Fab fragments (Boehringer Mannheim), 4× SSC, 3% BSA and 0.05% Tween 20 was applied to each slide. The slides were incubated at 37°C for 30 minutes in darkness. The slides were then washed three times in  $4 \times$  SSC, 0.05% Tween 20 in darkness at 42°C for 5 minutes each, before a brief wash in fresh deionized water. Samples were air dried before being mounted with Vectashield anti-fade mountant (Vectorlabs) containing 2 μg ml<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI) as a counter stain.

Repeat FISH experiments, duplicating specific chromosome delineation, were performed on the Landrace sperm to determine any variations in experimental and analytical procedures.

For tissue sections of testes, 12  $\mu$ l probe was applied to each tissue section and sealed with an 18×18 mm coverslip and rubber cement. Both the probe and testis sample were denatured at 85°C for 6 minutes and left to hybridize at 37°C in a humidified container for 2 days. The post-hybridization washes were performed as described previously for the spermatozoa with the following exceptions: the probe detection was performed using a 1:100 dilution of streptavidin conjugated with fluorescein (Amersham Biosciences RPN 1232) and the sections were mounted using Vectashield anti-fade mountant with propidium iodide (Vectorlabs). Extra propidium iodide was added to the mountant to give a final concentration of 7.5  $\mu$ g  $\mu$ l<sup>-1</sup>.

## Image capture and analysis

Spermatozoa were examined with a Leica epifluorescence microscope and observed under a 100× oil-immersion objective. Images were acquired with a CCD camera (Photometrics) and were pseudocoloured using Smart CaptureVP v1.4 (Vysis) providing a merged colour image. The positioning of whole chromosomes within the spermatozoa were analysed using PaintShop Pro 7. Various measurements were collected including the distance from the centre of the chromosome territory to the nearest peripheral edge of the spermatozoa and the length of spermatozoa for at least 50 sperm nuclei. The distances were normalized by dividing the distance from the centre of the chromosome territory to the nearest edge by the sperm length. Given that porcine sperm nuclei are virtually

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rectangular ('lozenge') in shape and have easily discernable head and tail regions; the length of the sperm could be divided by three, giving rise to three regions virtually equal in area. These regions were labelled anterior, medial and posterior.

The statistical significance of the results for the radial positioning from the same chromosome between different breeds of pig, for instance SSCX in Duroc, Landrace and Large White breeds of pig, was determined using one-way analysis of variance (ANOVA). Comparisons between different chromosomes within the same breed were analysed using Z-tests (http://www.le.ac.uk/biology/gat/virtualfc/Stats/ttest.html). The positioning of chromosome territories within each particular domain (i.e. anterior, medial or posterior region) of the spermatozoa was also recorded, as well as the packaging of chromosomes into either round or elongated territories. A  $\chi^2$  test was used to determine whether the positioning of chromosome territories between the anterior, medial and posterior sectors of the sperm nuclei were statistically significant.

The frozen porcine testes sections were imaged for two colours (red and green) using a BioRad MRC 600 confocal laser-scanning microscope with a helium neon laser and for three colours (blue, red and green) using a Leica TCS SP confocal laser-scanning microscope with an additional ultraviolet laser. Stacks of optical sections were collected at 1.5  $\mu m$  (BioRad) and 0.15  $\mu m$  (Leica TCS) intervals through the tissue. The stacks were viewed and analysed in 3D reconstructions using Imaris software. Chromosome position at the nuclear edge was scored in the 3D stacks.

#### Results

# Radial chromosome territory positioning in porcine sperm

Hybridized chromosome signals for each chromosome paint, in each breed of pig, were examined in at least 50 mature sperm nuclei. The exception was chromosome 12, whose signal was consistently too weak for analysis. All chromosomes were clearly organized into distinct chromosome territories within porcine spermatozoa (Fig. 1).

To compare the territorial positioning of chromosomes within spermatozoa with respect to having a peripheral, intermediate or interior location, the distance from the centre of the chromosome territory to the nearest peripheral edge of the spermatozoa was ascertained. Positioning of chromosome territories appears to be very similar between the three breeds of pig with chromosomes 2, 8, 11 and 15 occupying more peripheral locations within the sperm nuclei and chromosomes 9, 10, 14, X and Y within the interior (Fig. 2). Although chromosomes 9, 10, 14, X and Y all have interior locations, comparisons using Z-tests for two-sample means shows that chromosomes X and Y have a more central positioning than chromosomes 9, 10 and 14, because there is a highly significant difference (at least P=0.02247) for the normalized territory positioning for chromosomes X and Y, compared with chromosomes 9, 10 and 14. Interestingly, although the positioning of chromosome X is highly conserved in an interior location within sperm nuclei, this contrasts greatly with its positioning within stimulated lymphocytes, embryonic and adult fibroblasts and ex vivo adult mesenchymal stem cells, in which they occupy more-peripheral locations. This is mirrored by the Y chromosome, which is also more peripheral in stimulated porcine lymphocytes (our unpublished results). The territorial positioning of each chromosome was compared to the corresponding chromosome in each of the different breeds of pig via one-way ANOVA at a 95% confident level.

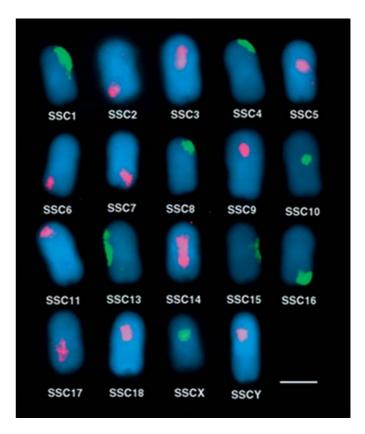
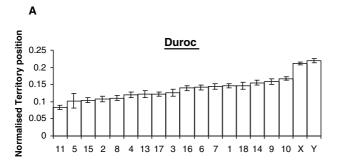
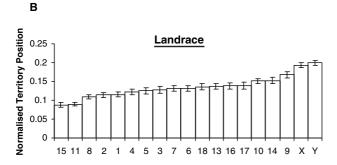
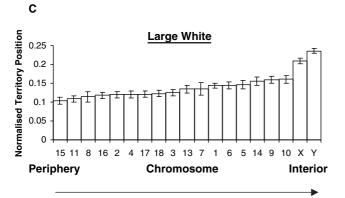


Fig. 1. Representative two-dimensional FISH images showing chromosome territories within Landrace porcine spermatozoa. Whole-chromosome-painting probes were either labelled with digoxigenin and detected via anti-digoxigenin conjugated to fluorescein isothiocyanate (green) or to biotin and detected using strepavidin conjugated to Cyanine 3 (red). The sperm nuclei are counterstained with DAPI (blue). Bar, 5 μm.

This determined whether the territories were randomly or nonrandomly distributed. Most chromosomes showed no significant differences between the means of the samples and were therefore non-random in position. Chromosomes 2-4, 6-9, 13-18 and X all had a null hypothesis (H<sub>0</sub>) that there were no differences between the means of the samples accepted at the 95% confidence level. However, when tested by one-way ANOVA, chromosomes 5, 10, 11 and Y showed differences between the means of samples for the different breeds at the 95% confidence level, and so the H<sub>0</sub> was rejected. Chromosomes 1 and 5 within Duroc, Landrace and Large White breeds of pig had significant differences between their means, indicating that they were randomly positioned between the different breeds. Nevertheless, for chromosome 1 the average normalized territory measurement was 0.146±0.009 in Duroc sperm, 0.116±0.0073 in Landrace sperm and 0.144±0.0067 in Large White sperm; so, for at least two breeds of pig, although the average value was significantly different, it was still similar (Fig. 2). It is clear that chromosomes exhibit distinct non-random radial organization within mature porcine spermatozoa and this positioning is not correlated with size, because there are small chromosomes found both at the nuclear edge and deep in the interior of the sperm nuclei.



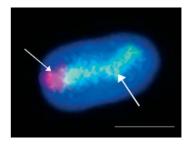




**Fig. 2.** Positioning of chromosome territories distributed radially in spermatozoa. Measurements were taken from the centre of a territory to the nearest edge. This measurement was normalized by dividing it by the length of the sperm. Data are plotted from the most peripheral chromosomes on the left-hand side to the most internal chromosomes on the right-hand side. (A) Duroc. (B) Landrace. (C) Large white.

# Morphology of chromosome-territory organization in sperm

The shape of chromosomes in sperm heads has been reported previously. Interestingly, chromosomes can adopt different morphologies that appear quite different to somatic cell chromosome territories. Sperm territories can be very condensed, organized into spherical structures (Fig. 3, large arrow) or less condensed in appearance and organized as elongated territories (Fig. 3, small arrow). Most chromosomes tended to have spherical territories, although chromosomes 1, 5 and 13 had high proportions of elongated territories (Table 1). Chromosomes 1 and 13 in Duroc sperm had 66% (P=0.025) and 54%, respectively, of their chromosome territories organized as elongated chromosomes. Interestingly, these two



**Fig. 3.** Representative image showing the morphology of both an elongated and a spherical territory in a Large White sperm head. The red SSC3 chromosome territory is packaged as a spherical territory and shown by the small arrow on the left. The larger arrow on the right highlights the green SSC4 chromosome territory that is organized in an elongated shape. Bar,  $5 \, \mu m$ .

chromosomes are the largest porcine chromosomes. Thus, size could be a determinant of how chromosome territories are packaged. However, SSC6 is the third largest chromosome but has few elongated territories, 16%, 8% and 16% in Duroc, Landrace and Large White breeds of pig, respectively (Table 1) and SSC 5 a more medium-sized chromosome has many elongated territories, 60%, 30% and 54% in Duroc, Landrace and Large White breeds of pig, respectively (Table 1). This indicates that some chromosomes are packaged differently to others, suggesting a difference in chromatin type or modification. Although, the presence of elongated territories is not correlated with the presence of CpG islands (McQueen et al., 1997). All of the X and Y chromosome territories were round, with the exception of X-chromosome territories in Landrace sperm, of which 2% were elongated.

#### Longitudinal positioning of chromosome territories

If the organization were random, as is apparently the case in chicken-sperm nuclei, approximately 33.3% of chromosome territories would be distributed equally within the three regions of each sperm nucleus. However, it was found that chromosomes occupy preferential locations along the sperm head (Table 2), especially in Duroc, but with Large White being closest to having a random longitudinal positioning of territories. Nonetheless, when comparing analogous chromosomes in the different pig breeds, some of the chromosomes demonstrate a high degree of variability for their longitudinal positioning (Table 2), for example chromosomes 13 and 15.

In Duroc, peripherally located chromosomes 8, 11 and 15 appear to be located preferentially at the anterior region of the spermatozoa. The more internally localized chromosomes 1, 10, 14, 18, X and Y tend to have a higher proportion of hybridization signals within the medial section of the spermatozoa, with far fewer being located in the posterior region. Because there is more DNA in the interior of the flattened sperm, there will be more chromosomes located there. This distribution is less obvious in the other breeds. It is the positioning of the sex chromosomes that is the most striking, with no chromosome territories being found within the posterior region for any breed and an exceptionally high proportion being found within the mid-section of the spermatozoa. Although a high proportion of the sex

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Table 1. Proportions of chromosome territories with an elongated or round shape in three breeds of porcine spermatozoa

		Duroc (%)			Landrace (%)		Large White (%)			
Chromosome	Elongated	Round	P	Elongated	Round	P	Elongated	Round	P	
1	66	34	0.025	52	48		17	83	0.0005	
2	24	76	0.001	14	86	0.0005	20	80	0.0005	
3	28	72	0.01	31	69	0.05	14	86	0.0005	
4	25	75	0.005	24	76	0.005	23	77	0.005	
5	60	40		30	70	0.025	54	46		
6	16	84	0.0005	8	92	0.0005	16	84	0.0005	
7	47	53		22	78	0.0005	28	72	0.01	
8	14	86	0.0005	17	83	0.0005	18	82	0.0005	
9	14	86	0.0005	22	78	0.0005	10	90	0.0005	
10	2	98	0.0005	0	100	0.0005	12	88	0.0005	
11	18	82	0.0005	6	94	0.0005	17	83	0.0005	
13	54	46		45	55		43	57		
14	22	78	0.0005	6	94	0.0005	14	86	0.0005	
15	26	74	0.005	28	72	0.01	52	48		
16	24	76	0.001	16	84	0.0005	23	77	0.001	
17	32	68	0.05	25	75	0.005	8	92	0.0005	
18	24	76	0.001	8	92	0.0005	15	85	0.0005	
X	0	100	0.0005	2	98	0.0005	0	100	0.0005	
Y	0	100	0.0005	0	100	0.0005	0	100	0.0005	

P values in the right-hand columns indicate where the proportions of the two types of chromosome organization are significantly different from an expected random organization of 50:50 using the  $\chi^2$  test.

chromosomes were positioned in the medial region of sperm nuclei, many of the territories were located at or on the anterior/medial interface, with most signal falling into the medial category (Fig. 4). There is no obvious correlation between the size of a chromosome and its anterior, medial or posterior positioning within the sperm nuclei.

# Sex-chromosome position in spermatocyte and spermatid cells

In order to determine the level of alteration to genome organization in sperm nuclei from the cells they originate from, we analysed chromosome positioning in sperm-precursor cells,

primary and secondary spermatocytes and spermatids (Fig. 5A-D). The sex chromosomes were analysed because they had the most interesting positions in sperm heads (i.e. they were the most internal and were located differently to normal somatic cells). We also analysed the positions of two autosomes (SSC5 and SSC13) to compare with the sex chromosomes. To assess chromosome positioning in sperm precursor cells we performed FISH on 3D preserved tissue sections of testis. After analysing the positions of the chromosomes in the three identifiable cell types (primary spermatocytes, secondary spermatocytes and spermatids), it was found that chromosome 5 was located at the nuclear edge in most of the three cell types (Fig. 5A,A'). However, chromosome 13 changed position

Table 2. Distribution of chromosome territories between the anterior, medial and posterior regions of spermatozoa in three breeds of pig

Duroc (%)				Landrace (%)					Large White (%)									
Chromosome	Anterio	or P	Medial	P	Posterior	P	Anterior	P	Medial	P	Posterior	· P	Anterior	P	Medial	P	Posterior	P
1	20		50	0.025	30		28		30		42		25		17	0.025	58	0.0005
2	40		30		30		48	0.05	10	0.0005	42		38		22		40	
3	34		49	0.025	17	0.025	29		49	0.025	22		29		32		39	
4	38		20		42		45		16	0.025	39		39		25		36	
5	16	0.025	54	0.005	30		0	0.0005	44		56	0.0005	15	0.01	50	0.025	35	
6	27		29		44		43		16	0.025	41		30		44		26	
7	6	0.0005	49	0.025	45		9	0.0005	22		69	0.0005	14	0.005	42		44	
8	74	0.0005	13	0.005	13	0.005	60	0.0005	15	0.01	25		54	0.005	28		18	0.05
9	48	0.05	36		16	0.025	39		49	0.025	12	0.005	32		20		48	0.05
10	16	0.025	84	0.000	5 0	0.0005	35		55	0.001	10	0.0005	10	0.0005		0.005	36	
11	88	0.0005	10	0.000	5 2	0.0005	67	0.0005		0.005	21		64	0.0005	5 17	0.025		0.05
13	54	0.005	34		12	0.005	55	0.001	27		18	0.05	24		46		30	
14	25		59	0.000	5 16	0.025	50	0.025	36		14	0.005	25		48	0.05	27	
15	66	0.0005	20		14	0.005	38		28		34		24		36		40	
16	43		49	0.025	8	0.0005	36		26		38		40		33		27	
17	18	0.05	46		36		31		34		35		20		29		51	0.01
18	30		52	0.01	18	0.05	24		45		31		27		40		33	
X	26		74	0.000	5 0	0.0005	40		60	0.0005	0	0.0005	35		65	0.000	5 0	0.0005
Y	6	0.0005	94	0.000	5 0	0.0005	31		69	0.0005	0	0.0005	51	0.01	49	0.025	0	0.0005

P values in the right hand columns indicate where the fractions scored of the three types of chromosome position are significantly different from an expected random distribution of 33.3:33.3:33.3 using the  $\chi^2$  test.

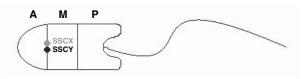
Table 3. Proportions of chromosome territories located at the nuclear periphery in primary spermatocytes, secondary spermatocytes and spermatid cells from porcine testis sections

Chromosome	Primary spermatocytes (%)	Secondary spermatocytes (%)	Spermatids (%)		
5	78	86	81		
13	51	64	100		
X	76	63	19		
Y	75	38	13		

radically during spermatogenesis, being at the nuclear edge in 51% of primary spermatocytes and 100% of spermatids (Fig. 5B,B'). The sex chromosomes also changed position radically but, in contrast to chromosome 13, were found more at the nuclear periphery in primary spermatocytes (76% SSCX and 75% SSCY) (Fig. 5C,C',D,D'). In secondary spermatocytes, 63% exhibited chromosome X at the nuclear edge and 38% had the Y chromosome at the nuclear edge. In spermatids, which have a smaller volume, the proportion of cells with X and Y at the nuclear edge was 19% and 13%, respectively (Table 3). This reveals that, as spermatogenesis is progressing through meiotic divisions to make spermatids, chromosomes are being repositioned; that is, chromosome 13 becomes more peripheral whereas the sex chromosomes become more internally located.

#### **Discussion**

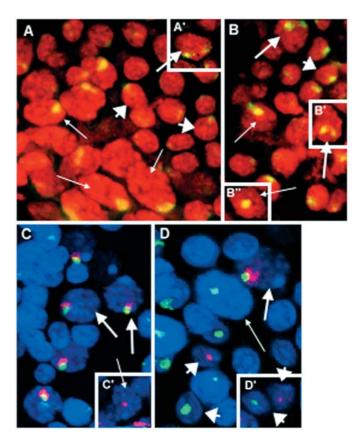
It is important to understand how the genome functions at fundamental stages in development and, to this end, we have analysed the spatial organization of the genome by chromosome positioning in sperm heads, spermatocytes and spermatids. We have determined that, within porcine spermatozoa, as with other mammals, the genome is highly organized into discrete chromosome territories that occupy non-random positions. This could have significant implications for the inheritance of nuclear organization and gene-expression patterns in early embryogenesis. Most interestingly, in our study, nuclear position of the sex chromosomes is completely different in sperm precursor cells in spermatogenesis (the spermatocytes) (Fig. 5) and in the cells that the two parental pronuclei will eventually give rise to, somatic cells (data not shown). The positioning of the sex chromosomes in primary spermatocyte nuclei is in agreement with others who have observed the XY body in these cells at the nuclear periphery, associated with the nuclear lamina (for a review, see Handel, 2004). Repositioning of the sex chromosomes appears to take place when the primary spermatocytes become secondary spermatocytes and then differentiate into spermatids. From our



**Fig. 4.** Schematic representation of a sperm nucleus showing the borderline anterior-medial position of the sex chromosomes SSCX and SSCY that is consistently observed in the spermatozoa from Duroc, Landrace and Large White breeds of pig. A, anterior; M, medial; P, posterior.

data, it appears that this is more rapid for the Y chromosome (Table 3). The opposite was seen for chromosome 13, which was repositioned more from the interior to the nuclear edge. This correlates with its position at least in Duroc sperm heads. Given our data, it seems that sperm nuclei have their chromosomal positions altered specifically for fertilization.

Chromosome positioning is yet to be established in early embryos, but chromosome positioning displayed in spermatozoa is quite different to that in porcine embryonic tissue culture cells (our unpublished results). This lends yet more credence to the concept that chromosome positioning is functionally significant in sperm nuclei. Interestingly, it has been demonstrated that round spermatid nuclei, in which sexchromosome position mirrors spermatozoa (this study), can be used successfully to make viable embryos by nuclear transfer into normal oocytes in mice and pigs (Sasagawa et al., 1998; Kim et al., 1999). However, secondary spermatocyte nuclei can also be used in this way to construct embryos with a similar success rate (Kimura and Yanagimachi, 1995). This might be due to the fact that the chromosome positioning seen in sperm



**Fig. 5.** Chromosome territories of porcine chromosomes 5 (A,A'), 13 (B,B',B''), X and Y (C,C',D,D'). Confocal optical mid-sections of frozen tissue sections of porcine testes are displayed with insets showing different cell types from other sections. Nuclei are counterstained with propidium iodide pseudocoloured in red (A,B) and the autosomes delineated in green. (C,D) Nuclei are counterstained with DAPI (blue), the X chromosome territories are in red and the Y chromosome territories are in green. Primary spermatocytes are identified by broader long arrows, secondary spermatocytes are identified by narrow long arrows and spermatids are identified by short, thick arrows.

heads is already being established as early as secondary spermatocytes in spermatogenesis (this study).

How might this complete relocation of chromosomes occur in spermatogenesis? Nuclei, including sperm nuclei, have several different proteinaceous structures like the nuclear envelope and the nuclear matrix. The nuclear envelope is made up of the nuclear membrane and complexes of integral membrane proteins (IMPs) such as the lamin-B receptor (LBR), lamina-associated proteins (LAPs) and nuclear lamins. Chromatin forms links with these structures, which stabilize it, anchor it and organize its distribution. All of these nuclear structures alter dramatically during spermatogenesis (Chen et al., 2001; Sousa and Carvalheiro, 1994; Alsheimer and Benavente, 1996; Alsheimer et al., 1998), possibly giving rise to altered chromosome positioning. LAP2\beta and lamin B1 are an IMP and a constitutively expressed lamin, respectively. Both these proteins bind chromatin and might be responsible for chromatin and chromosome localization to the nuclear periphery (Bridger and Bickmore, 1998). In spermatozoa precursor cells, LAP2β and lamin B1 concomittantly change their distribution. In round spermatids, the distribution of LAP2β changes from being around the circumference of the nuclei to being only at one half of the nuclear perimeter. Later, in elongated spermatids, it relocates again to the centriole pole. Lamin B1 behaves similarly, with a coincident temporal alteration in location (Alsheimer et al., 1998). This relocation of chromatin-binding proteins from the nuclear edge in spermatids could be why chromosomes normally found subjacent to the nuclear envelope change position. It might explain why they are released, but not their centralized location. Furthermore, protoamine 1, one of the proteins that complexes with DNA in sperm, has an affinity for LBR (Mylonis et al., 2004). Normally, in somatic cells, the chromatin-binding protein HP1 associates with LBR (Ye and Worman, 1996). This change could ultimately bring about a gross reorganization of sperm chromatin domains. The role of nuclear structure at the timing of this chromosomal repositioning will be investigated in future studies.

To the best of our knowledge, this is the most complete exposition of genome organization in the gametes of any organism. This is the first study that has assessed radial positioning in a small nucleus such as the sperm head for so many chromosomes and we have revealed preferential radial locations of chromosomes that are conserved between different breeds. Although some chromosome positions are shown to be different by statistical analysis in the three breeds, these chromosomes still maintain very similar locations within spermatozoa nuclei. For instance, Fig. 2 shows that chromosome 11 reproducibly occupies a very peripheral position within the spermatozoa, whereas chromosomes 10 and Y always occupy much more internal positions in all three breeds. An exception to this is chromosome 5, which resides in different locations within the different breeds (Fig. 2) but is mainly peripheral in primary and secondary spermatocytes and spermatids (Table 3).

Other studies have analysed chromosome position in sperm by dividing the sperm head into regions, namely the anterior, median and posterior regions. It has been found in human, rat and specific marsupial species that there is for the most part a non-random organization of chromosomes along the longitudinal axis. This has now been demonstrated for pig as well. In this study, the most convincing longitudinal positioning was demonstrated for the sex chromosomes. These chromosomes were most commonly located at the anterior-medial borderline. This is somewhat at odds with other studies for humans and monotremes, because these have found the X chromosome more towards the anterior of the sperm head (Greaves et al., 2001; Greaves et al., 2003; Hazzouri et al., 2000; Zalenskaya and Zalensky, 2004). However, it should be realized that we have localized the sex chromosomes to the border between the anterior and median regions. The sex chromosomes are also the chromosomes that are the most internal radially (i.e. they are the further from the nearest nuclear edge than any other chromosomes). This fits with the sex-chromosome distribution in marsupials, wombat and dunnart (Greaves et al., 2001; Greaves et al., 2003). This means that, in the pig, the sex chromosomes are probably some of the last chromosomes to be affected by their new, maternal, cytoplasmic environment after fertilization. After inclusion of the sperm nucleus after fertilization into the oocyte, the nuclear envelope breaks down, sperm chromatin immediately starts to decondense and associate with factors in the oocyte cytoplasm such as maternal histones (Wright, 1999) and nuclear structural components. It is highly likely that certain domains of chromatin are decondensed and remodelled at different times leading to different levels of control and consequently different gene expression patterns in the early embryo. Given the polar nature of the sperm, it seems reasonable to suggest that chromatin that is nearer the anterior edge would be under temporally different epigenetic control mechanisms to those at the posterior end. However, because the sperm heads of pigs and humans are not linear like those of birds and lower mammals, the radial position of the chromosomes might be at least as if not more important, with respect to the timing of chromatin remodelling and modification. That is, according to this theory, regions of chromatin around the nuclear edges are affected first by the maternal cellular environment and those nearer the anterior edge more than any other region at the nuclear periphery. So, chromosomes deep in the nuclear interior, such as X and Y, should be the last to respond to signals from the pig oocyte cytoplasm. After the chromatin remodelling by maternal factors - the paternal pronucleus is created by building a new nuclear envelope (Sutovsky and Schatten, 2001). This will comprise proteins not found in the sperm such as embryonic lamins, certain integral membrane proteins and nuclear matrix proteins, allowing new attachments to nuclear structure to occur so that the new male pronucleus genomic organization might be totally altered in the fertilized egg.

Demethylation of the paternal genome is initiated a few hours after entry into the oocyte, and remethylation occurs later (Barton et al., 2001). This is also a form of epigenetic control over the paternal genome that could be affected by chromosome location in the sperm nucleus. Another is DNA replication, which is initiated in sperm pronuclei at ~12 hours after fertilization (Wright, 1999). Chromosome position in the sperm head might influence where chromatin is positioned in the pronucleus and replication might be initiated at specific regions, leading to chromatin that could be classed as early and late replicating, and possibly associated with different pools or amounts of chromatin-binding proteins, setting up epigenetic

patterns in the paternal genome. The position of the X chromosome is intriguing because, in other cell types (our unpublished results), the X chromosomes are at the nuclear periphery. This is the same for the Y chromosome in stimulated lymphocytes (our unpublished results).

Even though most chromosomes are non-randomly distributed in sperm, this does not seem related to size, although there might be a slight correlation with the gene density of a chromosome. Compared with other species, few genes have been assigned for pigs, but a general understanding of the gene density of each chromosome can be determined by synteny comparisons between human chromosomes and via CpG island distribution. CpG islands tend to be located at the 5' end of certain tissue-specific genes and are present at the promoter regions of all housekeeping genes (Cross and Bird, 1995). They have been used to map genes and are synonymous with chromosomal areas of active genes. R-Banded CpG island patterns have been shown on porcine chromosomes 2, 3p, 5p, 6, 7, 10, 13, 14 and 17 (McQueen et al., 1997). Fig. 2 shows that these chromosomes displaying a CpG island R-banded pattern during FISH analysis (McQueen et al., 1997) are distributed throughout the peripheral and intermediate regions of the spermatozoa, with none of these chromosomes being found in the sperm interior. Although this is not direct evidence, it would put most CpG-island-carrying genes towards the sperm nuclear periphery, which might be important to subsequent gene-expression timetables.

There is no correlation between the size of a chromosome and their position within the spermatozoa. For example, chromosomes 11, 18 and Y are all very small but are generally located peripherally, intermediately and internally, respectively within the spermatozoa of all three breeds of pigs. The larger chromosomes 1, 6 and 13 all generally occupy intermediate positions within sperm nuclei from each pig breed studied.

This study and others have demonstrated that chromosomes are not distributed randomly within spermatozoan nuclei of human, pig, rat and some marsupial species. These results suggest that the non-random positioning of chromosomes is functionally important in the subsequent role of the paternal genome in development. We are presently looking at sperm pronuclei in fertilized pig oocytes and gametogenesis to test these hypotheses further.

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