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Meiotic studies in an azoospermic boar carrying a Y;14 translocation

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Abstract. A reciprocal translocation between the q arm of the Y chromosome and the q arm of chromosome 14 was identified in a young, phenotypically normal boar presenting azoospermia. Testicular biopsies were analyzed by classical histological and immunolocalization techniques, and by fluorescence in situ hybridization. Meiotic pairing analysis of 85 pachytene spreads showed the presence of an open structure corresponding to a quadrivalent formed by chromosomes 14, X, and the derivative chromosomes 14 and Y in 84.7% of the cases. In the remaining cases (15.3%), a 'tri-

valent plus univalent' configuration was observed. Immunolocalization of γ H2AX revealed the presence of this modified histone in the chromatin domains of unsynapsed segments (centromeric region of chromosome 14) and spreading of the γ H2AX signal from the XY body throughout chromosome 14 in 7.05% of the cells analyzed. The potential causes of the observed infertility, i.e. activation of meiotic checkpoints and/or silencing of genes necessary for the progression of meiosis, are discussed.

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The prevalence of Y-autosome reciprocal translocations in the general human population is very low (1 in 2000) (Nielsen and Rasmussen, 1976; Powell, 1984). This kind of translocation has been observed in both fertile and sterile males (Delobel et al., 1998). The effects on fertility mostly depend on the part of the Y chromosome translocated to the autosome. Translocations of the euchromatic part of the Y chromosome to non-acrocentric chromosomes, for instance, are frequently associated with azoospermia (Sun et al., 2005).

In addition, in reciprocal Y-autosome translocations, the autosomal parts of both derivative chromosomes are associated with the sex chromosomes that are transcriptionally inactive during prophase I (Monesi, 1965; Solari,

1974; McKee and Handel, 1993; Turner et al., 2002, 2005; Baarends et al., 2005). Several hypotheses have been proposed to explain the failure of meiosis as a result of this association.

According to different authors, this phenomenon could be due to partial reactivation of the sex body (SB) leading to the expression of some genes located on the X chromosome (Lifschytz and Lindsley, 1972), or to spreading of the SB inactivation towards the autosomal segments attached to the SB, without reactivation of the latter one (Jaafar et al., 1993).

Until now, very few reports on the analysis of meiosis in individuals carrying Y-autosome translocations have been published. However, the recent development of protein immunolocalization techniques on surface spread spermatocytes now permits accurate analysis of the early stages of meiosis (recombination and pairing of homologous chromosomes). Moreover, recent studies have indicated that meiotic silencing of unsynapsed chromatin (MSUC), including meiotic sex chromosome inactivation (MSCI), could be mediated through recruitment of the kinase ATR by BRCA1 followed by phosphorylation of the histone H2AX (Turner et al., 2005). Therefore immunolocalization

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of γ H2AX on surface spread spermatocytes seems to be a pertinent strategy to identify genetically silenced chromosomal regions during meiosis (Baarends et al., 2005; Turner et al., 2005, 2006).

To our knowledge, the use of such techniques to analyze meiotic synapsis and gene expression in spermatocytes of Y-autosome translocation carriers has been reported only twice (Sun et al., 2005; Sciarano et al., 2007).

The main observations of the authors were 1) gradual heterochromatinization of the autosome arms invading the sex body and 2) enlargement of the γ H2AX signals. Both were more consistent with the second hypothesis of a spreading of inactivation from the sex body to the autosomal parts.

Recently our group identified a Y-autosome translocation t(Y;14)(q1.1;q1.1) in the pig species (Ducos et al., 2007). We intend to improve our knowledge of the meiotic behavior of Y-autosome translocations by using a fluorescence immunocytogenetic approach to study this case.

Materials and methods

Animal material

The 6-month-old boar was recruited from the national systematic control program of young pedigree boars destined for artificial insemination centers (Ducos et al., 2007). Although it was phenotypically normal semen analysis revealed azoospermia.

Cytogenetic and molecular characterization

Classical cytogenetic analysis (GTG banding) allowed the identification of a reciprocal translocation t(Y;14)(q1.1;q1.1) (Ducos et al., 2007) (Fig. 1). This result was confirmed by dual color chromosome painting using chromosomes Y and 14 painting probes as previously described by Pinton et al. (2005).

Histological analysis

Specimens were routinely processed: tissue was fixed in 10% buffered formalin, embedded in paraffin wax, cut at 4 μ m and stained with haematoxylin and eosin.

Fluorescence immunostaining and fluorescence in situ hybridization

A cell suspension was produced by mechanic dissociation of testicular material and transferred to a centrifuge tube. After deposition of the seminiferous tubule remnants, the supernatant was centrifuged at room temperature at 600 g for 5 min. The pellet was washed in PBS and finally resuspended in a few drops of fresh PBS. Twenty microliters of cell suspension were mixed with 20 μ l of 0.05% Triton X-100 solution (prepared in distilled water) and spread on a microscope slide. After 10 min, the preparation was washed for 10 min by adding 60 μ l of 0.04% Photo-Flo (Kodak) solution and finally fixed with 120 μ l of fixative consisting of 1% formaldehyde, 0.016% Triton X-100, pH 10. After a further 10 min, the slides were rinsed in distilled water and air-dried at room temperature.

Immunolocalization of meiotic proteins was performed using primary antibodies at 1:100 dilution in PBT (1 $\times$ PBS, 0.15% BSA, 0.1% Tween 20) as follows: rabbit anti-SCP1, rabbit anti-SCP3, mouse anti- γ H2AX (Abcam, Cambridge, UK) and human anti-centromere (Antibodies Incorporated, Davis, CA, USA). The slides were incubated overnight at room temperature in a humid chamber. After three 5-min washes in PBS, 0.1% Tween, the secondary antibodies, i.e. Alexa 594-conjugated donkey anti-rabbit IgG, Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and AMCA-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laborato-

ries, Grove, PA, USA) were applied at 1:100 dilution for 2 h at 37°C. After three 5-min washes in PBS, 0.1% Tween and a brief rinse in distilled water, slides were air-dried and mounted with antifade solution (Vector Laboratories Inc., Burlingame, CA, USA). Capture and analysis of the surface spread spermatocytes were performed using the Cytovision FISH imaging system (Applied Imaging, Sunderland, UK).

After synaptonemal complex (SC) analysis, the same cells were subjected to fluorescence in situ hybridization using painting probes generated from flow-sorted or microdissected chromosomes X, Y and 14 (Yerle et al., 1993; Pinton et al., 2003). The probes were labeled with biotin (SSC14), digoxigenin (SSCY) or FITC (SSCX). Biotin was revealed by Alexa 594-conjugated streptavidin (Molecular Probes, Eugene, OR, USA). Digoxigenin was revealed by a mouse anti-digoxigenin antibody (Roche Diagnostic, Meylan, France) and an Alexa 647-conjugated donkey anti-mouse antibody (Molecular Probes, Eugene, OR, USA). The probe labeled with FITC was revealed by a goat anti-FITC antibody (Bethyl, Montgomery, Texas, USA) and an Alexa 488-conjugated donkey anti-goat antibody (Molecular Probes, Eugene, OR, USA). FISH signals of the same cells for which SCs had previously been analyzed were captured and evaluated.

Cot RNA experiment

A Cot RNA experiment was carried out before immunostaining of the γ H2AX protein (to preserve RNA integrity) according to Turner et al. (2005). The Cot DNA probe was produced from 400 ng of porcine Cot DNA (Applied Genetics Laboratory, Melbourne, FL, USA) labeled with biotin by Nick Translation (Roche Diagnostic, Meylan, France).

Results

Molecular characterization

Dual color chromosome painting confirmed the results of classical cytogenetic analysis (Fig. 1) and the presence of a small green signal (Y chromosome) on the derivative chromosome 14 proved the reciprocity of the exchange (Fig. 2, arrowhead). Moreover, as expected, the Y painting probe also labeled the pseudoautosomal region on the X chromosome p arm (Fig. 2, arrow).

This experiment clearly demonstrated that chromosome 14 was almost entirely translocated onto chromosome Y and that the small derivative chromosome was constituted by the centromeric region of chromosome 14 and a small Y chromosomal segment (Yq-ter chromosome segment).

Histology

Histopathological analysis showed a diffuse atrophy of the seminiferous tubules due to a complete arrest of spermatogenesis associated with a severe diffuse hyperplasia of the Leydig interstitial cells (Fig. 3a). At higher magnification, the epithelium of the tubules showed the presence of spermatogonia and primary spermatocytes in great number but no evidence of further cell maturation. Numerous abnormal cells and cellular debris were observed in the lumen (megakaryocytosis, multinucleated cells, monstrous cells) (Fig. 3b).

Immunolocalization and FISH analyses

SC analysis of 85 pachytene nuclei showed the presence of an open structure corresponding to a quadrivalent formed by chromosome 14, chromosome X, and the derivative chromosomes 14 and Y in 84.7% of the cases (Fig. 4).

Fig. 1. GTG-banded karyotype of the boar carrying a Y;14 translocation.

Fig. 2. Metaphase of the translocation-carrying boar after dual-color chromosome painting (Y probe is revealed in green and chromosome 14 in red). The arrowhead indicates the presence of Y chromosome material on the derivative chromosome 14 and the arrow the pseudoautosomal region at the extremity of Xp.

Fig. 3. Histological sections of testicular tissue. (a) Complete arrest of spermatogenesis with hyperplasia of the Leydig interstitial cells (×100). (b) Presence of spermatogonia and primary spermatocytes but no evidence of further cell maturation. Abnormal cells and cellular debris are visible in the lumen (×400).

A 'trivalent plus univalent' configuration was observed in the remaining cases (15.3%). The origin of the univalent (derivative chromosome 14) was confirmed by FISH experiments (Fig. 5a2–e2, univalent indicated by the arrowhead).

Immunolocalization of γ H2AX revealed the presence of this modified histone in the chromatin domains of unsynapsed segments (Fig. 5b1, b2, b3). FISH experiments permitted more accurate identification of these regions (Fig. 5c, d, e). In the majority of cells analyzed (92.94%), the histone accumulated on chromosomes X and Y and on the centromeric region of chromosome 14 in the quadrivalent configurations. Moreover, in the 'trivalent plus univalent' (derivative chromosome 14) configuration, a γ H2AX pos-

Fig. 4. Schematic drawing of the open quadrivalent.

itive signal was always observed on this latter chromosome (Fig. 5b2, arrowhead). Finally in 7.05% of the cells analyzed, a spreading of the γ H2AX signal from the XY body to the entire chromosome 14 was identified (Fig. 5b3, arrow).

Fig. 5. Spermatocytes after immunolocalization and FISH experiments. (a1–3) Immunolocalization of SCP3, SCP1 and centromeres. (b1–3) Immunolocalization of γ H2AX. (c1–3) FISH using chromosome Y probe. (d1–3) FISH using chromosome 14 probe. (e1–3) FISH using chromosome X probe.

Fig. 6. Cot RNA FISH experiment. Cot DNA positive signals (nascent transcripts) labeled in red and γ H2AX signal in green. Note the absence of Cot signals in the γ H2AX-positive area (arrow).

The Cot RNA FISH experiment carried out to analyze the transcription level of the cells revealed a lack of Cot probe signals in the γ H2AX domains suggesting transcriptional repression of these regions (Fig. 6, arrow).

Discussion

To our knowledge this is the first report of a Y-autosome translocation in pigs. In contrast, several Y-autosome translocations have been identified in humans, frequently associated with spermatogenesis impairment. In our case (azoospermic boar), histopathological analysis revealed that the meiotic process halted during the first division. Previous studies have shown that the arrest of spermatogenesis can be due to synaptic anomalies associated or not with chromosomal abnormalities (see e.g. Oliver-Bonet et al., 2005; Sun et al., 2005, 2007; Martin, 2006; Topping et al., 2006; Sciurano et al., 2007).

The aim of our study was to analyze the early stages of meiosis (using immunolocalization techniques) in order to identify the phenomena potentially responsible for the boar's sterility.

The use of immunolocalization techniques revealed some meiotic abnormalities since SC analysis demonstrated the presence of an open quadrivalent in most cells studied, but also a 'trivalent plus univalent' configuration in some cells (15.3% of the spermatocytes analyzed). The formation of this latter configuration was probably due to meiotic pairing impairments caused by the small size of the deriva-

tive chromosome 14. The presence of this univalent can result in a lack of tension of the kinetochores during metaphase I. This phenomenon can be detected by a specific meiotic checkpoint. Indeed, the 'spindle checkpoint' occurs at the metaphase I stage and blocks the metaphase-anaphase transition of cells presenting a defective spindle or misaligned chromosomes (Eaker et al., 2001). In a more general way, the observed meiotic pairing abnormalities (unsynapsed regions in the quadrivalent and 'trivalent plus univalent' configurations) can be detected by another checkpoint, the 'pachytene checkpoint', which occurs during prophase of the first meiotic division (Roeder and Bailis, 2000). This checkpoint may trigger an arrest of the meiotic division. In this case, the presence of unpaired regions (open quadrivalents) and of a univalent in the spermatocytes analyzed could explain the observed arrest of spermatogenesis in this boar.

Our analysis was completed by immunolocalization of the γ H2AX protein (phosphorylated form at serine 139 of the minor histone H2AX). This revealed an accumulation of the modified histone in the XY body as well as in the autosomal unsynapsed regions, i.e. the centromeric region of chromosome 14 and the derivative chromosome 14 in the quadrivalent and 'trivalent plus univalent' configurations. These observations are in agreement with the recent results of Scirano et al. (2007), i.e. presence of γ H2AX in the chromatin domains of the unsynapsed segments of the X and Y chromosomes as well as in some autosomal segments. Recent studies in mice have shown that γ H2AX-positive domains corresponding to unsynapsed chromatin regions are transcriptionally inactive (Baarends et al., 2005; Turner et al., 2005, 2006). We used Cot RNA FISH to confirm these results in pigs. Our data suggest that the accumulation of γ H2AX correlated with transcriptional silencing of unsynapsed autosomal regions could be responsible for the inactivation of genes located in the centromeric region of chromosome 14 that are crucial for meiotic division, thereby leading to spermatogenesis arrest through apoptosis of the concerned cells.

Recent data available through the pig genome sequencing project ('http://pre.ensembl.org/Sus_scrofa/index.html' – currently release 43 code – Nov 2006) (Hubbard et al., 2007) revealed that some genes involved in the meiotic process are located on chromosome 14 in the region corresponding to the chromosome 14 segment of the derivative chromosome 14. The first one is *CKS2* (cyclin-dependent kinase subunit 2) located around 400 kb from the centromere. Studies carried out in knockout mice for that gene showed that *Csk2* is involved in the meiotic process (Spruck et al., 2003). Indeed *Csk2*^{-/-} mice presented normal early meiotic progression but an arrest of spermatogenesis at metaphase I. The phenotype previously described in these mice is very similar to the observations in the azoospermic boar.

The second gene, located approximately 8 Mb from the centromere of chromosome 14, encodes the Kinesin-like protein KIF13B (Kinesin-like protein GAKIN) that plays a critical role in spindle function and chromosome segrega-

tion (Hanada et al., 2000). The inactivation of *GAKIN* may lead to the inability of chromosomes to segregate at the first meiotic division.

Consequently the meiotic arrest observed in our azoospermic boar could be explained by the inactivation of these two genes (*CKS2* and *GAKIN*).

Gene expression analyses of *CKS2* and *GAKIN* using quantitative RT-PCR will be carried out on RNA extracted from the testicles of the translocation-carrying boar and compared to results obtained from a normal individual. This study will be complemented by RNA FISH experiments using BAC clones containing these two genes.

In a limited number of cases (7.05%) the presence of γ H2AX has also been observed on the entire chromosome 14 suggesting a spreading effect of the transcriptional repression from the sex chromosome towards the autosomal translocated chromosome. These observations are coherent with a spreading of the SB inactivation towards the attached autosome segments (Jaafar et al., 1993) rather than gene activation on the X chromosome (Lifschytz and Lindsley, 1972).

Other consequences of meiotic disturbances have been observed in Y-autosome translocation carriers. Sun et al. (2005), for example, reported a decrease of the recombination rate in a man carrying a (Y;1) translocation. We tried to analyze the meiotic recombination using antibodies against MLH1 (Baker et al., 1996) but without success. No clear signals of the recombination foci were obtained with these antibodies. This suggests a need to improve the methodology (use of antibodies specific to other recombination proteins such as MLH3 or MSH4). Nevertheless, the occurrence of a similar phenomenon in our case cannot be excluded. Such a decrease in the number of recombination foci, i.e. future chiasmata, could be responsible for inappropriate orientation and segregation of the chromosomes during the first meiotic division. This meiotic disturbance may also be detected by the previously described meiotic checkpoints.

In conclusion our results show the interest and power of immunolocalization techniques for studying the early stages of meiosis. They open up a new way for identifying and understanding the mechanisms of meiotic disturbance. The identification of γ H2AX-positive domains in the spermatocytes of individuals exhibiting impaired spermatogenesis could be a first step in the identification of new genomic regions potentially carrying genes involved in the control of meiosis.

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