Gonadal expression of aromatase and estrogen receptor alpha genes in two races of Tunisian mice and their hypofertile hybrids

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SUMMARY

House mice (Mus musculus domesticus) in Tunisia consists of two races, one carries the 40-acrocentric standard karyotypes and the other one is a robertsonian race (2n=22) homozygous for nine centric fusions (Rb). The F1 hybrids between the two chromosomal races showed a significant decrease in reproductive success and litter size. Such results can be related to the formation of meiotic trivalent in the hybrids leading to the production of viable aneuploid gametes and post-zygotic elimination of embryos due to chromosomal non disjionction events at meiosis. Moreover, testicular

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histology of F1 and backcross males showed in some cases a breakdown in spermatogenesis. In both females and males, androgens but also estrogens play an important role in gametogenesis. In this study, we have studied aromatase and estrogen receptor alpha (ERα) gene expression in the gonads of the two parental races and their chromosomal hybrids. The results showed that aromatase and ERα mRNAs are expressed in hybrid males of inter-racial crosses (female22Rb×male40Std and female40Std×male22Rb) and in hybrid females of inter-racial crosses (female22Rb×male40Std) as in the two parental races. However, in hybrid females of inter-racial crosses (female40Std×male22Rb) the amount of aromatase transcripts decreased sharply suggesting that this gene is involved in the breakdown of hybrid fertility in females, but not in males. However, in hybrid males, a putative post-translational modification of this enzyme, in terms of activity, should be verified. Reproductive Biology 2007 7 2:143–162.

**Key words:** transcripts, aromatase, ER alpha, gonads, mouse, infertile hybrids

**INTRODUCTION**

Chromosomally mediated speciation is a process whereby fixation of chromosomal rearrangements initiates and contributes to divergence and reproductive isolation between populations [24, 27, 42]. This model has several requirements: i/ hybridization between karyotypically divergent populations leads to unfit hybrids, ii/ the level of post-mating isolation due to chromosomal heterozygosity partially blocks sufficient gene flow to facilitate genic divergence, and iii/ pre-mating isolating mechanisms evolve through selection for reinforcement and bring speciation to completion [40]. In Tunisia, two chromosomal races of house mice exist. The first one has accumulated nine pairs of fusions (2n=22 chromosomes; 22Rb race) and is restricted to the central region of the country where it is sympatric with the standard morph (2n=40, 40Std race) which occurs all over the country [34]. Chromosomal differentiation in the house mouse, *Mus musculus domesticus*, through the accumulation of centric or Robertsonian (Rb)
fusions has been used to illustrate the chromosomal model of speciation [4, 42]. Within the distribution area of ancestral allacrocentric mice (2n=40), Rb populations carrying one to nine pairs of Rb fusions occur in geographically separate clusters of related races each differing by the number and type of fusions [2]. Saïd and Britton-Davidian [31] reported that the Tunisian 22Rb race is characterized by a patchy distribution as it is present only in urban centres surrounded by the standard morph. The latter occupies peripheral zones as well as rural villages, whereas outside the area of sympatry, it lives in both types of habitat. Contact between Rb and all-acrocentric mice yields chromosomal hybrids which show a decrease in reproductive success and litter size.

Such results can be related to the formation of meiotic trivalent configurations in the hybrids leading to the production of viable aneuploid gametes and post-zygotic elimination of embryos due to chromosomal non disjunction events during meiosis. Moreover, histological analysis of testes showed that the tubular diameter of seminiferous tubules, the cellular differentiation and germ cell density were reduced. Exfoliation and necrobiosis were also usually observed [33]. In female hybrids, the total number of follicles and of primordial follicles significantly reduced and the number of corpora lutea increased [9]. The degree of this disturbance was unrelated to the level of chromosomal heterozygosity suggesting that genetic incompatibilities between the two genomes are involved in addition to aneuploidy.

In addition to gonadotropins and testosterone, numerous intra-testicular factors including estrogens play a crucial role in the development and maintenance of spermatogenesis [5, 30]. Several lines of evidence have conclusively shown that estrogens are produced in the male gonad and are involved in the regulation of testicular development and functioning [7, 26]. The irreversible biosynthesis of estrogens from androgens is catalyzed by cytochrome P450 aromatase (P450arom), a product of a unique gene called \textit{Cyp19} which is located in the endoplasmic reticulum membrane of various tissues [39]. Although estrogens have been regarded as female hormones, they are now known to have profound effects on the male reproductive genital tract. Aromatase has been found in meiotic and post
meiotic germ cells predominantly in spermatids of mouse [25], rat [22] and human immature germ cells and spermatozoa [8, 20].

However, the role of estrogens in male reproduction is not fully understood and therefore extensively studied. Estrogens exert their effects through specific estrogen receptors (ER; [7, 18]). In the male reproductive tract, ERα has been shown to be strongly expressed in the Leydig cells, the epididymis and efferent ductules [18]. Expression of ERβ mRNA and protein was demonstrated in mouse Leydig cells and elongated spermatids as well as in developing rat Sertoli cells and type A spermatogonia and in adult rat Sertoli cells, pachytene spermatocytes and round spermatids [7, 35].

Recent investigations of mice deficient in ERα (ERαKO; [14]) or aromatase (ArKO; [28]) have provided direct evidence of the physiological role of estrogens in male reproductive organs. The ERαKO males were infertile due to inhibited luminal fluid reabsorption in the head of the epididymis leading to altered seminiferous tubules, decreased spermatogenesis and infertile sperm production [17]. The ovaries of ERαKO females contained cystic hemorrhagic follicles with few, if any, granulosa cells. A few primary follicles but no corpora lutea were present in the ovary [23]. The ArKO male mice showed a decrease in the number of round spermatids, an enhancement of apoptosis and impaired sexual behaviour which induced infertility [29]. Female ArKO mice are infertile following a disruption of the folliculogenesis and an inability to ovulate, as indicated by a lack of corpora lutea [3].

The gametogenesis is a complex, polygenic phenomenon which depends on the interactions of several genes distributed in all the genome, and ArKO mice gonadal observations show some similarities with the Tunisian chromosomal hybrid mice [38]. Therefore in the current paper, we have analyzed the expression of aromatase and ERα genes in the Tunisian parental mice of the two chromosomal races and their hybrids in order to explain the abnormal gametogenesis in the hybrids [33].
MATERIALS AND METHODS

Animals
The mice were caught during two separate periods (October-November 2002; November-December 2004) using traps placed in houses, shops, farm buildings, gardens and cultivated fields in different localities of Monastir (Tunisia). Mice were kept with access to food and water ad libitum under standard laboratory conditions at 20°C with 12 hours of day-light. Three series of reciprocal crosses were set up in the laboratory:

i/ intra-racial: 22Rb×22Rb: (7 females, 7 males),
40Std×40Std: (17 females, 17 males),
ii/ inter-racial: 40Std (17 females, 17 males)×22Rb
(17 females, 17 males).

Inter-racial crosses were established from the first generation progeny of the two types of intra-racial crosses using wild house mice live-trapped in Monastir. We have used 76 wild and laboratory hybrid mice (tab. 1).

Table 1. Chromosomal analyses of mice

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Diploid number (2n)</th>
<th>The chromosomes implicated in Robertosonian fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>22</td>
<td>2 Rb (1.11); 2 Rb (2.16); 2 Rb (3.12); 2 Rb (4.6); 2 Rb (5.14); 2 Rb (7.18); 2 Rb (8.9); 2 Rb (10.17); 2 Rb (13.15)</td>
</tr>
<tr>
<td>11</td>
<td>40</td>
<td>All chromosomes are acrocentric</td>
</tr>
<tr>
<td>39</td>
<td>31</td>
<td>1 Rb (1.11); 1 Rb (2.16); 1 Rb (3.12); 1 Rb (4.6); 1 Rb (5.14); 1 Rb (7.18); 1 Rb (8.9); 1 Rb (10.17); 1 Rb (13.15)</td>
</tr>
<tr>
<td>1 male</td>
<td>23</td>
<td>1 Rb (1.11); 2 Rb (2.16); 2 Rb (3.12); 2 Rb (4.6); 2 Rb (5.14); 2 Rb (7.18); 2 Rb (8.9); 2 Rb (10.17); 2 Rb (13.15)</td>
</tr>
<tr>
<td>1 female</td>
<td>23</td>
<td>1 Rb (1.11); 2 Rb (2.16); 2 Rb (3.12); 2 Rb (4.6); 2 Rb (5.14); 2 Rb (7.18); 2 Rb (8.9); 2 Rb (10.17); 2 Rb (13.15)</td>
</tr>
<tr>
<td>1 female</td>
<td>23</td>
<td>1 Rb (5.14); 2 Rb (1.11); 2 Rb (2.16); 2 Rb (3.12); 2 Rb (4.6); 2 Rb (7.18); 2 Rb (8.9); 2 Rb (10.17); 2 Rb (13.15)</td>
</tr>
<tr>
<td>1 male</td>
<td>24</td>
<td>1 Rb (1.11); 2 Rb (4.6); 1 Rb (5.14); 2 Rb (2.16); 2 Rb (3.12); 2 Rb (7.18); 2 Rb (8.9); 2 Rb (10.17); 2 Rb (13.15)</td>
</tr>
</tbody>
</table>
Chromosome preparation
Chromosomes were prepared from yeast-stimulated bone marrow cells using the air-drying method [21]. Diploid number was determined by examining up to five well-spread metaphases under a Zeiss Axiophoto microscope. Chromosomes were identified after G-banding, following the Seabright procedure [36] and the Cowell nomenclature [12] from photographs of 3-5 metaphase plates per individual.

RNA extraction
Total RNA from testes and ovaries was extracted using the guanidium thiocyanate-derived method [11]. Briefly, after centrifugation the cell pellets were homogenized on ice in 1 M Tris buffer containing guanidium thiocyanate (4 M). The RNA was isolated with a phenol-chloroform-isoamyl alcohol acid solution. It was precipitated twice from the aqueous phase with isopropanol, washed with 75% ethanol, dried on a speed-vac and dissolved in diethylpyrocarbonate-treated water and then stored at -80°C. The purity of the RNA samples was checked spectrophotometrically by measuring the optical densities at 260 and 280 nm and evaluating the ratio 260/280 nm. The quality of the RNA samples was visualized under UV transillumination after running on a 1.5 % agarose gel stained with ethidium bromide.

RT-PCR assay
Total RNA (2 µg) was reverse-transcribed to first-strand cDNA as follows: 1 h at 37°C with 200 IU M-MLV-RT (Promega, Charbonnières-France), 500 µmol/l dNTP, 0.2 µg of oligo-dT (12-18mers) and 24 IU RNasin in a final volume of 40 µl, then 5 min at 94°C. The cDNAs were further amplified by PCR using selected oligonucleotides [22]. PCR was performed in the presence of 1.5 mM MgCl₂, 200 µM dNTP, 1.5 IU Taq polymerase and 50 pmol of the forward and reverse primers (Life Technology, Eragny-France) in a final volume of 50 µl. All primers have been chosen in different exons in order to eliminate any potential contamination by genomic DNA. The selected PCR primers and expected length of the resulting PCR products as well as the cycle profiles are listed in Table 2.
For all PCR amplifications, negative (water only) and positive control (human granulosa cells) were included. All cDNA fragments were run on a 2% agarose gel stained with ethidium bromide and visualized under UV transillumination.

Table 2. Primer sequences used for RT-PCR and cycle profiles of different primer pairs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatase</td>
<td>5’-ACTTCTCGTGCGAGAGGTATCCAG-3’</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td>5’-AGCCCTTTTGGCTTTGGGCCGCCGC3’</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor alpha</td>
<td>5’-AATGAAATGGGTGCTTCAGG-3’</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>5’-ATAGATCATGCCGGTCAG-3’</td>
<td></td>
</tr>
<tr>
<td>Beta actin</td>
<td>5’-GACATCAAAGAGAGAAGCTGTGC-3’</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>5’-TAGGAGCCAGAGCAGTAATC-3’</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cycle profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatase</td>
<td>95°C/30 s, 56°C/30 s, 72°C/1 min</td>
</tr>
<tr>
<td>Estrogen receptor alpha</td>
<td>95°C/30 s, 60°C/30 s, 72°C/1 min</td>
</tr>
<tr>
<td>Beta actin</td>
<td>95°C/30 s, 60°C/45 s, 72°C/1 min</td>
</tr>
</tbody>
</table>

**Semi-quantitative RT-PCR**

In order to quantify the transcripts of aromatase and ERα in gonads, we have determined the optimal conditions for the RT-PCR and used actin as a housekeeping gene. As an example, the kinetics of PCR amplifications of aromatase, ERα and actin for female mouse are presented in Figure 1. The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Gels were photographed using photoprint Vilbert Lourmat system and analyzed with NIH image computer program (http://rsb.info.nih.gov/nih-image).
Fig. 1. Kinetics of PCR amplification; A/ aromatase gene expression in wild females: 2n=22 and 2n=40; B/ estrogen receptor alpha gene expression in two wild females 2n=40; C/ beta actin gene expression in wild females: 2n=22 and 2n=40. Arrows indicate the number of cycles for quantification of the PCR products.
**Statistical analysis**
Results are means±SEM. The Student’s *t*-test was used to compare the mean values and significance was accepted at *p*<0.05 (Stawords, Brain Power Inc, Calabasas, CA, USA).

**RESULTS**

**Cytogenetic characterization of mice**
The cytogenetic analysis (G-banding) showed that the mice with 2n=40 belong to the standard race (40Std) with 40 all-acrocentric chromosomes including 19 pairs of autosomes and a pair of sexual chromosomes X and Y.

The mice with 22 chromosomes belong to the Robertsonian race (22Rb) described before [31] and are characterized by the same nine pairs of Robertsonian fusions of the standard acrocentric chromosomes karyotype. This combination of fusions, specific of the Tunisian Rb system called “Monastir” implies all chromosomes except pair 19 and sexual chromosomes: Rb (1.11), Rb (2.16), Rb (3.12), Rb (4.6), Rb (5.14), Rb (7.18), Rb (8.9), Rb (10.17) and Rb (13.15) (tab. 1; [32]).

**Aromatase transcripts in gonads of two chromosomal races of mice and their hybrids**
Aromatase expression was examined in testes and ovaries of wild mice of the two chromosomal races, 22Rb (10 males and 8 females) and 40Std (2 males and 8 females), and their first generation laboratory hybrids 2n=31 (18 males and 18 females; tab. 1). Aromatase was present in various preparations of wild and hybrid testes (fig. 2A) as well as ovaries (fig. 2B). Using a semi-quantitative approach, aromatase and beta actin were shown to be present in different preparations of wild and hybrid testes (fig. 3A) although in lanes 2 and 4 a slight degradation of RNA was observed. Similarly, aromatase and actin were present in wild and hybrid ovaries (fig. 4A) although in lane 2 (hybrid female) a decrease in aromatase expression was detected.
Fig. 2. Expression of aromatase gene after RT-PCR amplification in two chromosomal races of mice and their hybrids; A/ wild and hybrid males; RNA extracted from testes of: wild 40Std - lanes 1, 2, 3, 4; wild 22Rb - lane 5; laboratory hybrids (2n=31) - lanes 6, 7, 8; natural hybrid (2n=24) - lane 9; positive control (human granulosa) - lane 10 and negative control (water) - lane 11; B/ wild and hybrid females, RNA extracted from ovaries of: wild 40Std - lane 1; wild 22Rb - lane 2; laboratory hybrids (2n=31) - lane 3; natural hybrid (2n=23) - lane 4; natural hybrid (2n=23) - lane 5; positive control (human granulosa) - lane 6 and negative control (water) - lane 7; M corresponds to DNA ladder (100 bp)
Fig. 3. A/ Semi-quantitative RT-PCR of aromatase and beta actin products in wild and hybrid males; RNA extracted from testes of: hybrids (2n=31) - lanes 1, 2, 3; wild 22Rb - lanes 4, 5 and wild 40Std - lane 6; B/ densitometric analysis of aromatase/actin mRNA ratio in different populations of wild and hybrid male mice; NS: not significant
Fig. 4. Semi-quantitative RT-PCR of aromatase and beta actin products in females of two chromosomal races of mice and their hybrids; A/ RNA extracted from ovaries of hybrid females (2n=31): F22Rb×M40Std - lane 1; F40Std×M22Rb - lane 2; RNA extracted from ovaries of wild females: 40Std - lanes 3, 4 and 22Rb - lane 5; B/ densitometric analysis of aromatase/actin mRNA ratio in the different populations of wild and hybrid mice; NS: not significant
Aromatase transcripts in gonads of hybrid mice (F40Std × M22Rb and F22Rb × M40Std)

Hybrid females (2n=31), a result of two crosses, demonstrated a significant difference in the amount of aromatase transcripts. Hybrid females of the cross (F40 × M22) were characterized by either a decrease or absence of aromatase transcripts (fig. 5A). Conversely, no great variation was observed in the expression of aromatase between hybrid males from the two cross types (fig. 6A).

There is no difference in the amount of aromatase transcript between male parental races and male hybrids (fig. 3B) as well as between separate hybrids of the two inter-racial crosses (fig. 6B). The aromatase/actin ratio showed a significant difference only between female hybrids (fig. 5B).

Fig. 5. Semi-quantitative RT-PCR of aromatase and beta actin products in hybrid females. A/ RNA extracted from ovaries of hybrid females (2n=31): F22Rb × M40Std - lanes 1, 2, 3, 7, 9, 10; F40Std × M22Rb - lanes 4, 5, 6, 8 and negative control (water) - lane 11; M corresponds to DNA ladder (100 bp); B/ densitometric analysis of aromatase/actin mRNA ratio in the different populations of hybrid mice; **p<0.01
Fig. 6. Semi-quantitative RT-PCR of aromatase and beta actin products in hybrid males. A/ RNA extracted from testes of hybrid males (2n=31): F22Rb×M40Std - lanes 1, 3 and F40Std×M22Rb - lanes 2, 4, 5, 6; B/ densitometric analysis of aromatase/actin mRNA ratio in the different populations of hybrid mice; NS: not significant
Detection of estrogen receptor transcripts in wild and hybrid mice

Estrogen receptor alpha mRNA was present in testes and ovaries of the two races and in their F1 hybrids (fig. 7). The differences between hybrid mice (male or female) from the various crosses were not significant (data not shown).

Fig. 7. Detection of estrogen receptor alpha gene in hybrid females and males; A/ RNA extracted from testes of hybrid males (2n=31) (F40Std×M22Rb): lanes 1-4 and negative control (water) - lane 5; B/ RNA extracted from ovaries of hybrid females (2n=31): (F22Rb×M40Std) - lanes 1, 5; (F40Std×M22Rb) - lanes 2, 3, 4, 6; negative control (water) - lane 7; C/ RNA extracted from: ovaries of hybrid females (2n=31) (F22Rb×M40Std) - lanes 1, 2, 5, 6; testis of hybrid male (2n=31) (F22Rb×M40Std) - lane 3; testis of hybrid male (2n=31) (F40Std×M22Rb) - lane 4; negative control (water) - lane 7

DISCUSSION

For a decade it has been demonstrated that aromatase is expressed not only in testicular somatic cells but also in various germ cells [7]. Although estrogens have been considered to be female hormones, they are now...
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known to have an important role in male reproductive functions via the presence of estrogen receptors ubiquitously present in testicular cells and all along the genital tract [8]. Herein, we wanted to determine if aromatase and ERα could be putative candidates to explain some disorders observed in gametogenesis of hypofertile hybrids coming from two Tunisian chromosomal races of house mice.

The counter-selection of the Tunisian mouse hybrids which seems to be stronger than in those between chromosomal races in Europe is not due to the presence of a particular type of fusion [9]. This selection is partially the result of a decreased fertility which is due not only to the presence of aneuploid gametes following the formation of atypical meiotic figures and chromosomal nondisjunction but also to genic disorders. Saïd et al. [33] and Chatti et al. [10] observed a decreased fertility or total sterility in mouse chromosomal hybrids which was related to major disturbances in the differentiation of the germ cells resulting in a blocking of gametogenesis at early stages of meiosis. Moreover, several reports on the fertility of the chromosomal hybrids showed that the degeneration of the spermatogonia well precedes the pairing of the homologous chromosomes [37].

We have observed that the levels of mRNA coding for both aromatase and ERα in testes of the two chromosomal races and their hybrids of first generation F1 (2n=31) and several generations (2n=23; 2n=24) are of the same magnitude. In females, the ERα expression was identical in the two chromosomal races and their hybrids (fig. 7). However, aromatase transcripts are better expressed in the female hybrids resulting from the inter-racial crosses (F22Rb×M40Std) than in those from reciprocal crosses (fig. 5). In the female hybrids of the reciprocal inter-racial crosses (F40Std×M22Rb) either a very weak expression or an absence of aromatase transcript was recorded. In females without aromatase expression the infertility status is well-known. Several studies in mammals have established that testes represent a source of estrogens [6, 22, 25]. The decrease in fertility in the chromosomal hybrids seems not to be due to a disturbance in aromatase gene expression; however, it is possible that the P450arom transcripts detected are translated into an inactive protein following a post-translational modification. To exclude
this possibility, the biological activity of the aromatase must be checked in these hybrids.

In the ArKO mice which are initially fertile, progressive infertility appears in the animals aged of 4 months [15, 19]. This anomaly which concerns all one year-old animals is marked by a stop in spermatid maturation associated with a reduction in their number and an increase in apoptosis. In addition, Honda et al. [19] and Robertson et al. [29] reported that the reduced fertility observed in these ArKO mice could be due to deterioration in sexual behavior which is coherent with the crucial role played by estrogens in the development and initiation of the sexual behavior in males. Conversely, the transgenic mice which express aromatase are infertile and develop tumours of the Leydig cells [16].

Several lines of evidence have suggested that, in addition to estrogens, mouse salivary androgen-binding protein might be responsible for reproductive isolation between subspecies of the house mouse Mus musculus by mediating sexual selection [1, 41]. This hypothesis resulted from genetic, evolutionary and behavioural studies of the gene Appa encoding the alpha subunit, common to all forms of this dimeric protein [13].

In conclusion, we have shown that aromatase gene is very weakly expressed in the mouse hybrid females (F40Std×M22Rb) possibly resulting in reduced female fertility. In males, however, the aromatase gene expression is probably not involved in the breakdown of fertility although the hypothesis of a putative post-translational modification of this enzyme in hybrid males deserves to be verified. We know that reproduction is a complex and polygenic phenomenon and depends on interactions of genes distributed in all the genome. Therefore, the analysis of other markers such as the estrogen receptor β or testosterone and gonadotropins levels will be helpful to understand the mechanisms and the origin of sterility in the Tunisian mouse hybrids.

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