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Investigating mouse sexual dimorphism at the molecular and histological levels

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Abstract

In mammals, the profoundly different pathways of male and female sex development are decided by a genetic coin toss; at fertilization either an X or Y chromosome is inherited from the father to unite with the X chromosome, which is always inherited from the mother. These differences begin to unfold during fetal development, when the TDY (Testis determining factor on Y chromosome) is activated in males and acts as a switch that diverts the fate of the undifferentiated gonadal primordia, the genital ridges, towards testis development. If this does not occur, alternative molecular cascades drive the genital ridges toward ovary development. In this paper, we present data obtained from conducting a series of experiments using the mouse model, designed to review the differences between male and female gonads at the molecular and histological levels. This project-based approach is intended for an undergraduate 14-week course (laboratory component of Developmental Biology) at American University in Cairo. Over the course of the semester, we examined the differential expression of Zfy (zinc finger Y-chromosomal protein), Zp3 (zona pellucida glycoprotein 3) and Cobra1 (cofactor of BRCA1) genes. While the expression of Zfy-gene, which is required for spermatogenesis, is restricted to the testes, the expression of the Zp3-gene, which is a structural component of the zona pellucida that surrounds oocytes, is limited to the ovaries. The gene Cobra1, on the other hand, is expressed in a wide variety of tissues including ovaries and testes. As illustrated in this paper, the project culminates in presenting our results in a publication-manuscript format, thus demonstrating our ability to assemble data into a coherent story, analyze and discuss the outcome of the experiments and set plans for future research. We believe this practice prepares us to contribute to prospective scientific investigations.

1. Introduction

In mammals the sex of the organism is determined after fertilization; however, it becomes apparent only during fetal development, when the gonads begin to differentiate as ovaries or testes after a considerable period of sexual ambiguity. All secondary sexual dimorphisms are thought to follow from the differentiation of the gonads and their acquisition of endocrine function. Decades ago, it was shown that the development of ‘femaleness’ represents the default state, and that the development of ‘maleness’ is linked to the presence of the Y chromosome (Jost, 1953). Years later, it was proven that not the whole Y chromosome is necessarily required for initiating the development of ‘maleness’, but a single copy gene named Sry (sex-determining region Y) is responsible for that (Gubbay et al. 1990).

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Throughout the semester, the laboratory exercises in the developmental biology course at the American University in Cairo allowed us to examine the differences in the sex development process in both male and female mice phenotypically and through studying the histology of both types of gonads (Amleh et al. 2010). Furthermore, at the molecular level we demonstrate the presence of the Y-chromosome in male but not female mice and the differential expression of two sex-specific genes, which contribute to the gonad development. The first examined gene is \( Zfy \), once considered as the testis determining factor (Page et al., 1987), which is located on the short arm of Y chromosome and required for spermatogenesis (Peng et al., 2011), and the second is the gene \( Zp3 \), a structural component of the zona pellucida that surrounds oocytes and is located on the long arm of chromosome 7 (Rankin et al., 1996). The association between the expression of \( Zfy \) or \( Zp3 \) and the sex of the mouse was further emphasized by demonstrating similar expression patterns of \( Cobra1 \), which encodes a transcription regulator, in both sexes irrespective of the tissue assayed. \( Cobra1 \) encodes for the cofactor of \( Brca1 \) and is a member of the negative elongation complex, which is known to stall RNA polymerase II preventing the elongation of the transcripts of certain genes (Narita et al., 2003). Moreover, protein level of COBRA1, which is expressed in all assayed tissues of both male and female mice, is analyzed via Western blotting. Experiments were carefully designed to fit in a three-hour class period.

2. Materials and Methods

2.1. Adult sexual dimorphism at the phenotypic level and tissue harvesting

Sexually mature (adult) mice, ~8 weeks old were used. Each mouse was phenotypically sexed by inspection of external genitalia. The ano-genital distance, which is the distance between the anus and the external genitalia (penis/vagina), was measured and the presence or absence of nipples was noted. Thereafter, liver, kidneys, ovaries and testes were retrieved for DNA extraction, RNA extraction and histological analysis.

2.2. Histological analysis of gonads

One mouse gonad from each gender/sex was fixed in 4% paraformaldehyde at 4° C overnight to be followed by a series of graded ethanol washes before passing it through xylene and finally embed it in paraffin. Thin sections (10 \( \mu \)m thick) sections were obtained, mounted on glass slides and left to dry at room temperature. Hematoxylin and Eosin (H & E) staining technique was used to study the histological structures of the gonads.

2.3. Molecular analysis of extracted tissues

2.3.1. DNA extraction, analysis and genotyping

15-25 mg of kidney tissue was homogenized in PBS using a pestle and QIAamp® DNA Mini kit was used for DNA extraction. Assessment of DNA samples was done by two approaches; spectrophotometer and gel electrophoresis. Concentration was calculated: OD260 nm (of the sample) x 50 x dilution factor = concentration of DNA (\( \mu \)g/ml). Genotyping was performed using \( Zfy \) primers while \( \beta\text{-actin} \) primers were used as the positive control.

2.3.2. RNA extraction and analysis

70 mg of tissue were used for RNA extraction using TRIZOL® according to manufacturer’s protocol. The RNA samples were assessed for quantity and quality. Concentration was calculated: OD260 (of the sample) x 40 x dilution factor = concentration of RNA (\( \mu \)g/ml). RNA integrity was also tested via gel electrophoresis.

2.3.3. Synthesis of cDNA and PCR

cDNA was synthesized by RevertAid™ First Strand cDNA Synthesis Kit in the presence of reverse transcriptase (RT) according to manufacturer’s protocol. To examine the expression of the selected genes \( -Zfy, Zp3 \) and \( Cobra1-\)
in cDNA of different tissues, PCR was carried out using primers for these genes and Gapdh primers were used to test for the endogenous control.

2.3.4. Protein analysis

Proteins were extracted using Nonidet-P40 (NP40) lysis buffer while protein concentration was determined using Thermo Scientific Pierce BCA protein kit. Immunoblotting including detection was conducted using Western Breeze kit®, Invitrogen according to manufacturer’s instructions.

2.4. Embryonic Gonadal development

Uteri of pregnant female mice were removed by separation from the mesometrium, and embryos were freed from the uterus by cutting transversely between implantations. The embryos were observed under a dissecting microscope and staged based on the development of the limbs (Rugh, 1991). Gonads of embryos were examined.

3. Results

3.1. Adult sexual dimorphism at the phenotypic level

Male external genitalia were observed to consist of a penis, scrotal sacs and an anus (Figure 1A). The male ano-genital distance was longer compared to female ano-genital distance as shown in Table 1. After dissecting the male abdomen, two oval testes were noted.

<table>
<thead>
<tr>
<th>Table 1. Ano-genital distance in adult male and female mice</th>
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<tr>
<td>Ano-genital distance (cm)</td>
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Female external genitalia were observed to consist of urethral orifice, vaginal orifice and anus (Figure 1B). After dissecting the female abdomen, two ovaries extending from the oviduct connected to the uterine horns were observed. Also, while nipples were absent in the male abdomen, female abdomen showed 2 rows of nipples 5 on each side.

Figure 1. External genitalia of male and female adult mice. (A) Male mouse abdomen top to bottom (blue arrows): penis, scrotal sacs and anus. (B) Female mouse abdomen top to bottom (blue arrows): urethral orifice, vaginal orifice and anus; (orange arrows): two nipples.

3.2. Histological analysis of the adult mouse gonads

The differences in adult male and female gonads at the histological level are illustrated in Figure 2. While germ cells -although not seen at this magnification- are housed in seminiferous tubules of the testis, they are contained by follicles in the ovary. Within these structures germ cells grow and mature.
Figure 2. Histology of adult-mouse gonads. (A) Testicular cross section representing a number of seminiferous tubules composed of germ and Sertoli cells. (B) Ovarian cross section representing oocytes within follicles at different stages of development as well as the remnants of an ovulated follicle (corpus luteum).

3.3. Molecular analysis of adult sexual dimorphism

3.3.1. Quantitative and Qualitative DNA Analyses

DNA was quantified by using the UV spectrophotometer (Table 2). The ratio of OD260/OD280 indicated that the DNA is of high quality. Furthermore the genomic DNA integrity is preserved as indicated in Figure 3.

<table>
<thead>
<tr>
<th></th>
<th>OD260 nm</th>
<th>OD280 nm</th>
<th>OD260/OD280 Ratio</th>
<th>Calculated concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sample</td>
<td>0.242</td>
<td>0.129</td>
<td>1.87</td>
<td>121 µg/ml</td>
</tr>
<tr>
<td>Female sample</td>
<td>0.098</td>
<td>0.05</td>
<td>1.96</td>
<td>49.0 µg/ml</td>
</tr>
</tbody>
</table>

Figure 3. DNA quality. Lane 1: 1 Kb ladder, Lane 2: Male Kidney DNA, Lane 3: Female Kidney DNA and Lane 4: positive control.

3.3.2. PCR genotyping analysis

The chromosomal sex was determined using PCR genotyping technique. Genomic DNA was assayed for the presence of Y chromosome using primers that are specific for Zfy sequences (Figure 4). Zfy gene is located on the Y chromosome and hence present in male DNA (Figure 4, lane 3) but not in female DNA (Figure 4, lane 4).
3.3.3. Quantitative and Qualitative RNA Analyses

RNA was first quantified by using UV spectrophotometer and the concentration was accordingly determined (Table 3). RNA purity on the other hand was deduced from the OD260/280 ratio (Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Ovary</th>
<th>Testis</th>
</tr>
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<tbody>
<tr>
<td>OD 260 nm</td>
<td>1.59</td>
<td>0.2</td>
<td>2.06</td>
</tr>
<tr>
<td>OD 280 nm</td>
<td>0.81</td>
<td>0.1</td>
<td>0.93</td>
</tr>
<tr>
<td>Ratio OD 260 /OD 280</td>
<td>1.95</td>
<td>2.02</td>
<td>1.88</td>
</tr>
<tr>
<td>Concentration in µg/µl</td>
<td>2.54</td>
<td>0.32</td>
<td>3.06</td>
</tr>
</tbody>
</table>

RNA integrity was assessed by gel electrophoresis. Intact total RNA when run on agarose gel is expected to display two clear bands one corresponding to 28S and the other to 18S rRNA (ribosomal RNA). In intact total RNA, the intensity of the 28S band is anticipated to be approximately twice as that of the 18S rRNA band (Figure 5, lane 4). However, in degraded RNA the 2:1 ratio of high quality RNA will be replaced by smeared appearance (Figure 5, lanes 2 & 3). The size of 28S rRNA band is 4.7 kb (4700 bp) and that of 18S rRNA is 1.9 kb.

3.3.4. Gene expression profile of Zfy, Zp3 and Cobra1

Gene expression pattern of Zfy, Zp3 and Cobra1 was examined by RT-PCR using gene-specific primers. As indicated below, transcripts generated by Zfy gene were detected in testicular RNA (Figure 6A), Zp3 gene in ovarian RNA (Figure 6C, lane 9), and Cobra1 gene in RNA extracted from all tested tissue samples whether from XX or XY mice (Figure 6B lanes 2, 3, & 4). The presence of a band with the right molecular size in the negative control sample (Figure 6B lane 5) is indicative of cross contamination. Gapdh was used as an endogenous control (Figure 6C, lanes 1 to 5).
Figure 6. Gene expression profile. PCR was done for cDNA samples by using the following: (A) Zfy primers (amplicon size 618 bp): Lane 1: 100 bp ladder, Lane 2: negative control, Lane 3: positive control, Lane 4: Male Kidney cDNA, Lane 5: Ovary cDNA, Lane 6: Testis cDNA. (B) Cobra1 primers (amplicon size 492 bp): Lane 1: 100 bp ladder, Lane 2: Testis cDNA, Lane 3: Ovary cDNA, Lane 4: Male Kidney cDNA, Lane 5: negative control, Lane 6: positive control. (C) For the first 5 lanes Gapdh primers were used (amplicon size of 949 bp): Lane 1: Male Kidney cDNA, Lane 2: testis cDNA, Lane 3: negative control, Lane 4: Ovary cDNA, Lane 5: positive control, Lane 6: 100 bp ladder. For the last 5 lanes Zp3 primers were used (amplicon size 600 bp): Lane 7: Male Kidney cDNA, Lane 8: Testis cDNA, Lane 9: Ovary cDNA, Lane 10: negative control and Lane 11: positive control.

3.3.5. Detection of mouse COBRA1 by Western blotting

Consistent with expression at the RNA level COBRA1 protein is present in the analyzed tissues (Figure 7B). Protein concentration in both liver and kidney tissues were obtained relative to a carefully constructed standard curve (Figure 7A). We also assayed for α-TUBULIN (Figure 7C) to ascertain that comparable amounts of protein were loaded for the recognition of COBRA1 protein.

![Figure 7. Protein analysis. (A) Protein quantification by preparing a standard curve for several BSA concentrations. (B) Western blot for COBRA1 which is of size 65.5 KDa and (C) Western blot for α-TUBULIN which is of size 55 KDa (loading control).](image)

3.3.6. Morphological analysis of gonadal development in embryos

Embryonic gonadal/structural organization was not apparent in each of the examined ages. Based on embryonic limb morphology/development, we retrieved embryos at around ~15 dpc (Figures 8A & 8B) and others from a different pregnant female at ~11 dpc (Figure 8C). The general structure of the gonad especially that of the testis, was easily recognized at 15dpc (data not shown).

![Figure 8. Extracted mouse embryos. (A) Embryo at ~E15: the whole view of mouse in petri dish under light microscope. (B) Embryo at ~E15: a closer view magnification to investigate limb development. (C) Embryo at ~E11.](image)

4. Discussion

The presented work is the outcome of a project that was conducted throughout the Development Biology course at the American University in Cairo. The main goal was to investigate the sexual dimorphism in mammals using the mouse model. It is well accepted and represented in figure 4 that the presence of the Y chromosome dictates the differentiation of the gonad into testes while its absence results in development of ovaries (Gubbay et al., 1990,
Koopman et al., 1991). As gonadal sex is established, it directs phenotypic sex (Figure 2). Therefore a chromosomal XY mouse gives rise to a male phenotype (Figures 1A & 2A, Table 1) whereas an XX gives rise to a female phenotype (Figure 1B & 2B, Table 1). DNA and RNA extractions were analyzed before molecular assays were conducted (Figure 3 & 5, Table 2 & 3). Throughout the male and female reproductive life the expression of certain genes is required to ensure the production of gametes that are competent for fertilization thus for reproducing the species. Consistent with such notion and with previous reports the expression of Zfy is shown to be restricted to the testis whereas Zp3 expression is specific to the ovary (Figure 6). Whether the expression of Cobra1 (Figure 6 & 7), which is a transcriptional regulator, plays a role in controlling differential gene expression remains to be determined. Observing testicular organization at 15dpc but not at 11dpc, reveals that the gonadal organization, which is secondary to the molecular switch, has been already established (Wilhelm et al., 2007), and embryos were extracted at those particular ages (Figure 8).

5. Conclusion

Gonad development, because of its dimorphic nature, is a fascinating example of organogenesis, in which one common primordium has the potential to develop into either of two morphologically and functionally different organs, testis or ovary. In this case we can further study molecular mechanisms and networks of gene regulation that result in the formation of functional organs, which most likely can be translated into other systems of organogenesis.

Acknowledgements

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References