

## TTY2 genes deletions as genetic risk factor of male infertility

F. Shaveisi-Zadeh<sup>1</sup>, R. Alibakhshi<sup>2</sup>, R. Asgari<sup>3</sup>, Z. Rostami-Far<sup>4</sup>, M. Bakhtiari<sup>5</sup>, H. Abdi<sup>6</sup>, A. Movafagh<sup>1##</sup>, R. Mirfakhraie<sup>1##</sup>

<sup>1</sup>Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Biochemistry, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

<sup>3</sup>Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

<sup>4</sup>Molecular Pathology Research Center, Imam Reza Hospital, Kermanshah University of Medical sciences, Kermanshah, Iran

<sup>5</sup>Department of Anatomical Sciences and Biology, Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

<sup>6</sup>Department of Urology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

Correspondence to: [abolfazlmovafagh110@gmail.com](mailto:abolfazlmovafagh110@gmail.com) or [reza\\_mirfakhraie@yahoo.com](mailto:reza_mirfakhraie@yahoo.com)

Received September 17, 2016; Accepted February 20, 2017; Published February 28, 2017

# These authors contributed equally to this work

Doi: <http://dx.doi.org/10.14715/cmb/2017.63.2.8>

Copyright: © 2017 by the C.M.B. Association. All rights reserved.

**Abstract:** Y chromosome has a number of genes that are expressed in testis and have a role in spermatogenesis. TTY2L12A and TTY2L2A are the members of testis transcript Y2 (TTY2) that are Y linked multi-copy gene families, located on Yp11 and Yq11 loci respectively. The aim of this study was to investigate frequency of TTY2L12A and TTY2L2A deletions in azoospermic patients compared with fertile males. This study was performed on 45 infertile males with idiopathic azoospermia without any AZF micro deletions (group A), 33 infertile males with azoospermia which do not screened for AZF micro deletions (group B) and 65 fertile males (group C), from October 2013 to April 2015 in west of Iran. Polymerase chain reaction (PCR) method was used for detection of TTY2L12A and TTY2L2A gene deletions in studied groups. No deletions were detected in normal fertile males of group C. 1 out of 45 azoospermic males of group A (2.22%) and 3 out of 33 azoospermic males of group B (9.09%) had TTY2L2A deletion ( $p=0.409$  and  $p=0.036$  respectively), also 1 out of 45 azoospermic males of group A (2.22%) and 4 out of 33 azoospermic males of group B (12.12%) had TTY2L12A deletion ( $p=0.409$  and  $p=0.011$  respectively). None of azoospermic males in Group A and B had deletions in both genes. Our data showed significant correlation between non-obstructive azoospermia and TTY2L12A and TTY2L2A deletions. Thus, it seems that TTY2L12A and TTY2L2A deletions can consider as one of the genetic risk factors for non-obstructive azoospermia.

**Key words:** Azoospermia; TTY2; Male infertility; Deletion.

### Introduction

Infertility constitutes a global medical problem. According to World Health Organization (WHO) definition, infertility is a disease of the reproductive system means the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). Infertility is classified as primary or secondary, depending on the presence of a previous history of pregnancy. Approximately 15 to 20 percent of all couples are infertile. It is suggested that 30-40% of infertile couples suffer from male factor infertility, while 30-40% from female factor infertility and the rest from unexplained or combination factors involving both partners (2).

Male infertility is a multifactorial syndrome includes a wide variety of disorders and despite great advances in the field of infertility, 30 percent cases of male infertility cases are still defined as idiopathic (3). More than half cases of male infertility are related to impairment of spermatogenesis. Sperm abnormalities are a critical factor in male infertility and include azoospermia (not having any measurable level of sperm in semen) oligospermia (low concentration of sperm) asthenospermia (reduced sperm motility) teratospermia (reduced nor-

mal sperm morphology) and aspermia (complete lack of ejaculation) (4).

Several factors are involved in male infertility which can be broadly divided into non-genetic factors (include varicocele, ejaculatory duct obstruction, sperm agglutination, sexual dysfunction and hormonal imbalance), epigenetic and genetic factors (5). According to latest findings, genetic factors account for 10-15% of male infertility and include chromosomal aberrations and single gene mutations. About 30% cases of male infertility associated with impaired spermatogenesis (azoospermia or oligospermia) have no known cause, the condition that defined as idiopathic male infertility (6).

The Y chromosome is one of smallest chromosomes spans more than 59 million base pairs and represents almost 2 % of the total DNA in human genome. Y chromosome micro deletions are one of the the most frequent genetic causes of male infertility especially in patients with oligo and azoospermia (7). However, the molecular mechanisms of spermatogenesis defects in males and its relationship with small deletions in the Y chromosome are not completely understood (8). Results of different studies shows that the probability of mutations in Y chromosome increased because of rapid division of germ cells during the fetal and puberty phases, also

as all genes on the Y chromosome are haploid, defects in these single genes are more probably to have effects, even it is compensated due to the presence of multi copy genes on the Y chromosome (9).

Polymerase chain reaction (PCR)-based analysis of Y-chromosome sequence-tagged sites (STS) in infertile males indicate the occurrence of micro deletions in a region of Y chromosome which is not detectable by classical cytogenetic techniques. This led to the identification of 3 non overlapping loci in Yq11 region named the azoospermia factor (AZFa, AZFb, and AZFc) which carries genes involved in control of spermatogenesis (10).

The genes located in AZF regions (such as BPY2, DAZ, RBM2, RBM1, USP9Y and CDY) codes proteins involved in the regulation of gene expression, RNA metabolism, packaging and transport to cytoplasm and RNA splicing (11). Deletions in AZF region arise from recombination between repeated sequences within the Y chromosome and can cause severe spermatogenic defects ranging from non-obstructive azoospermia (10-15%) to oligospermia (5-10%) (12). Many genes expressed during spermatogenesis possess are responsible for encoding proteins that are involved in sperm production. The deletion in these genes may lead to infertility or sub fertility in males. However, these known genes does not explain the cause of the all cases of idiopathic male infertility and the role of the other genes located on Y chromosome remains unknown (13).

Studies performed on Y specific testis expressed cDNA library by use of Y-linked cosmids lead to identification of a Y-linked multicopy gene family called testis specific transcripts (TTY) (14). TTY is a large gene family with at least 26 copies, located on both arms of Y chromosome and divided into at least 14 subfamilies based on similarity between sequences (15). The function of TTY gene family is not clear so far. TTY2 gene family is one member of TTY genes presenting moderate to high homology to each other (59% to 99%). One member of TTY2 gene family called TTY2L2A mapped on Yq11.2 within AZFc region, while another member of this family, TTY2L12A located in Yp11 region in distal short arm of the Y chromosome near the telomere (16). Despite of many studies carried out on the role of Y-linked genes such as USP9Y and RBM, there are no many studies in literature about TTY2 genes (17). Therefore, this study, attempts to clarify the relationship between deletions of TTTY2L2A and TTTY2L12A, with male infertility which is performed on two groups of patients with idiopathic non-obstructive azoospermia from central and western parts of Iran.

## Materials and Methods

### Study design

This case-control study was carried out on infertile

**Table 1.** The sequence of primers used in this study.

Row	Name	Sequence	Annealing temp	Product length
1	TTTY2L12A-F	5-CAGACTGTGAGTTGGTTCTG-3	54°C	233 bp
2	TTTY2L12A-R	5-TATGTGAGAGAGACCCTGTG-3		
3	TTTY2L2A-F	5-CCTATCTGAGCAGGTACTTTAC-3	56°C	178 bp
4	TTTY2L2A-R	5-GTGTCATCTGTCTTTCTCAGTG-3		

males referred to the Motazedi infertility teaching clinic of Kermanshah University of Medical Sciences. After detailed clinical examination, patients were subjected to semen, endocrinological and cytogenetic examinations.

The diagnosis of azoospermia was established on the basis of the independent analysis of at least two semen samples according to the World Health Organization guidelines (4). Serum follicle stimulating hormone (FSH), Luteinizing Hormone (LH) and Testosterone levels were determined by immunoassay (Cobas e411 analyzer, Roche Diagnostics, Mannheim, Germany).

All subjects were of Iranian ethnic origin and this research and patients care were assigned on the basis of National/International metabolic programs and approved according to official Iranian law and regulations by the Institutional Review Boards of each participating teaching and referral hospital. Also our study complies with the Declaration of Helsinki.

Azoospermic patients having any cryptorchidism, congenital absence of vas deferens, seminal tract obstruction, varicocele, testicular tumors or undergoing chemotherapy and radiotherapy were excluded from this study and finally, 78 males with idiopathic non obstructive azoospermia who were within the age range of 25-38years were included in this study during the period from November 2015 to June 2016. All patients have normal karyotype.

### Studied groups

A total of 78 patients were included in the present analysis. Patients were divided into two groups: Group A consisted of 45 idiopathic azoospermic males with central Iran origin. These patients have been studied in terms of AZF micro deletions of the Y chromosome (according to EAA/EMQN guidelines) and have no deletion. Group B consisted of 33 idiopathic azoospermic males with western Iran origin which have not been studied in terms of AZF micro deletions, and Group C consisted of 65 males with western and central Iran origin who had fathered at least two children considered as control group.

### DNA analysis

1 ml of blood samples were taken from all the patients and controls and collected in tubes containing EDTA. Genomic DNA was isolated from peripheral blood leukocyte using QIAGEN DNA extraction kit and stored in -20°C. The PCR comprised a total volume of 25 µL, of which 10 µL was the master mix (2X) (Amplicon, Denmark), 1 µL of each primer, 100 ng of genomic DNA, and 8 µL of enzyme-free water. The primer sequences are shown in Table 1. Amplification was carried out for 30 cycles in a Eppendorf Mastercycleras follows: denaturation at 94°C for 45 s, annealing at 54°C for TTTY2L12A (56°C for TTTY2L2A) for 30 s and extension at 72°C for 45 s. Amplification cycles were

proceeded by initial denaturation for 5 min at 94°C and final extension at 72°C for 6 min. In each PCR reaction, DNA from normal fertile males was used as normal control, while water served as negative control. The PCR products were separated on a 1% agarose gel prepared in 1× TBE buffer containing GelRed dye by electrophoresis. A 100bp DNA marker (Fermentas) was loaded with PCR products to confirm the band size. A positive result was scored when the amplification product of expected size was obtained. Deletion of PCR fragments was confirmed by three repetitions.

### Statistical analysis

SPSS version 16 software was used for the statistical analysis. All data were initially checked for normality of distribution with the Kolmogorov–Smirnov test and were found normally distributed. Data are presented as the mean ± standard deviation (SD), median and percentage for categorical variables. One-way ANOVA was used to compare the means between groups. The Fisher's exact test were used for evaluate differences between subjects with detected deletions in TTY2 genes for each group. Pearson's correlation analysis was used to study the association between quantitative variables.  $P < 0.05$  was considered statistically significant.

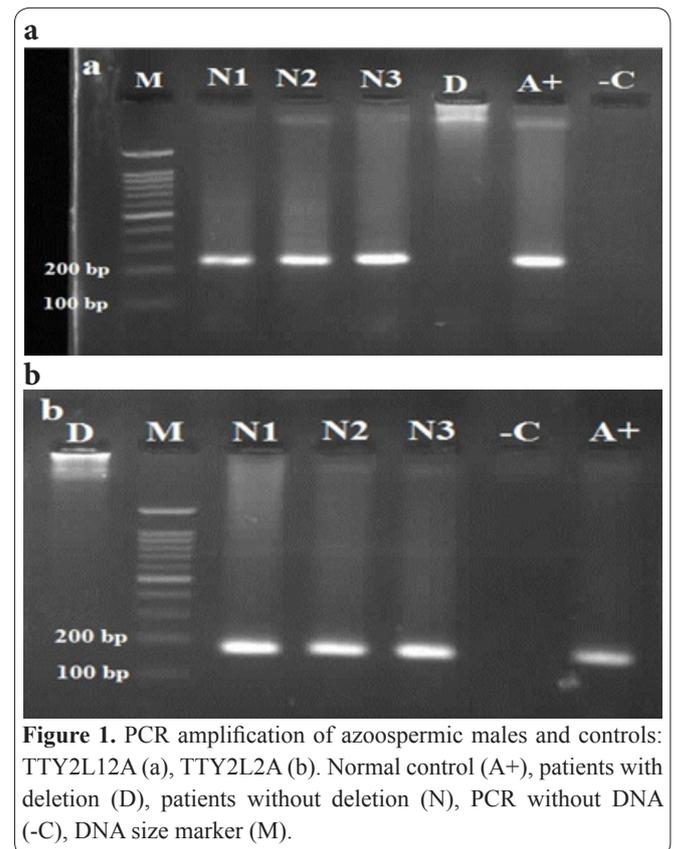
### Results

In this study, only azoospermic males with Iranian origin were included. The patients were divided into two groups. Group A consisted of 45 azoospermic males who have no microdeletions in the AZF region and group B consisted of 33 azoospermic males who not investigated for AZF micro deletions. 65 fertile males considered as control group (group C). All patients had normal karyotype (46, XY) analysis and all of the patients and the controls have no family history. Table 2 shows the information on age and hormonal levels of patients and controls.

After performing PCR with specific primers of TTY-

2L2A and TTY2L12A genes, no deletions were found in a sample of 65 normal fertile controls, however, in group A and B, samples of some patients were not amplified, which indicates deletion of TTY2L2A and TTY2L12A genes (Table 3).

Deletions of TTY2L2A gene were seen in 2.22% and 9.09% males of group A and B respectively. Also TTY2L12A genes were deleted in 2.22 % and 12.12 % of males of group A and B respectively. Deletion of both TTY2L2A and TTY2L12A genes were found in none of the patients. Frequency of deletions of both TTY2 genes and images of PCR amplification and gel electrophoresis are shown in Table 3 and Figure 1.



**Figure 1.** PCR amplification of azoospermic males and controls: TTY2L12A (a), TTY2L2A (b). Normal control (A+), patients with deletion (D), patients without deletion (N), PCR without DNA (-C), DNA size marker (M).

**Table 2.** Information on age and hormonal levels of men participating in the study.

	Group A (n=45)	Group B (n=33)	Group C (n=65)	Normal Ranges
Age (years)	32.4±6.4	31.5±7.2	34±5.9	
<b>Hormone Levels</b>				
Testosterone	4.9±2.8	5.1±2.5	5.4±2.6	2.8-8 ng/ml
LH	12.1±6.3	11.5±5.7	4.9±2.1	1.7-8.6 mIU/ml
FSH	29.8±1.6	27.6±2.5	8.2±5.3	1.5-12.4 mIU/ml

**Table 3.** Frequency of deletions of TTY2 genes in studied groups.

	Number	Deletions identified in genes		p-value
		Yes	No	
<b>TTY2L2A</b>				
Group A	45	1(2.22%)	44(97.78%)	0.409
Group B	33	3(9.09%)	30(90.91%)	0.036
Group C	65	0(0%)	65(100%)	Reference
<b>TTY2L12A</b>				
Group A	45	1(2.22%)	44(97.78%)	0.409
Group B	33	4(12.12%)	29(87.88%)	0.011
Group C	65	0(0%)	65(100%)	Reference

## Discussion

Male infertility has attracted a great deal of attention from many genetic and biology researchers and until now, many genes have been identified which are involved in spermatogenesis process that their mutation can affect male fertility potential (2,5). The Y chromosome carries genes, which are needed for sex determination and spermatogenesis. However, all genes required for sperm production are not located on Y chromosome. Indeed around 30 genes from about 200 genes involved in spermatogenesis are located on Y chromosome and others are located on X or autosomes (9).

TTY genes are a large gene family and have at least 14 subfamilies, located on different parts of Y chromosome which have no similar sequences on the other chromosomes. TTY genes are RNA coding, but have no certain open reading frame (ORF) (14). It is believed that TTY genes expressed in testis exclusively, although few studies showed that their expression is not restricted to testis tissue, also their roles in spermatogenesis are not quite clear (16). TTY2 gene family is one of the TTY genes distributed in large areas of both arms of Y chromosome and have medium to high homology with each other (59% to 99%) (15). Higher homology increases the chance of homologous recombination events between the TTY2 genes, which disrupt their functions and can lead to infertility. Indeed, Y chromosomes mutations frequently occur due to recombination within highly repetitive DNA sequences during the male meiosis (12).

The results of our study showed that in control group, none of the males had deletion in TTY2 genes, but some patients in group A and B had deletions in both TTY2 genes; so it seems that, these genes are involved in spermatogenesis process, but the mechanism is unclear.

The TTY2L2A gene has 26.5 kb length and located in the Yq11 region. The Yq11 region is the location of three separate sub-regions called AZFa, AZFb and AZFc. Deletions in AZF regions of Y chromosome are the most common known molecular genetic cause of spermatogenic failure. TTY2L2A specifically is located in AZFc region near important genes such as BPY2, TSPY, PRY and DAZ which have a vital role in spermatogenesis process (15). In our study, TTY2L2A gene were deleted in only one out of 45 azoospermic patients (2.22%) of group A which were not statistically significant. The patients of this group were screened and have no AZF micro deletion. In patients of group B, three out of 33 patients (9.09%) have a deletion of TTY2L2A which were statistically significant. The patients of this group not screened for AZF micro deletions. Since the frequency of deletions of TTY2L2A gene in group B are more than group A and this gene located in AZFc region, so any possible rearrangements which lead to the deletion of TTY2L2A, should involve AZFc region (which contain the candidate genes of spermatogenesis). To the best of our knowledge, the study of Yapijakis and colleagues on Greece infertile males published in 2014 is the only clinical study performed on relationship between of TTY2 gene deletions and male infertility. In that study, 11.8% of patients with idiopathic severe oligo and azoospermia showed a deletion in TTY2L2A, however patients were not investigated in terms of AZF

micro deletions (16).

The TTY2L12A gene has 29 kb length and located in the Yp11 region. Except SRY which is a very important gene, deletions occurring in the short arm of Y chromosome are not frequent. However, interestingly in our study TTY2L12A gene deletion was detected in four out of 33 azoospermic patients (12.12%) of group B ( $P>0.05$ ) and one out of 45 patients (2.22 %) of group A ( $P<0.05$ ). Also in study of Yapijakis and colleagues, 29.4% of severe oligo and azoospermia patients have a deletion in TTY2L12A which are in accordance with results of our study in terms of higher rate of TTY2L12A to TTY2L2A deletions (16).

In our study, none of the patients have deletions in both studied TTY2 genes, while TTY2L2A and TTY2L12A are very far apart. In study of Yapijakis and colleagues, overall 8.8 % of patients had deletions in both studied TTY2 genes. In this study, total deletions of TTY2L2A and TTY2L12A genes in group A and B were 4.44 % and 21.21 % respectively; therefore, it is recommended that this survey will be repeated in other ethnics and populations (15,16).

The high percentage of deletions and sequence similarity in TTY2L2A and TTY2L12A genes suggest that TTY2 genes probably have a similar and important role in complex spermatogenesis process, but the understanding of mechanism is still very poor and details are not known. Since the TTY2L2A and TTY2L12A genes expressed in testis, have no clear open reading frame and do not code any proteins, it seems that they regulate spermatogenesis through the mechanisms such as RNA interference (RNAi), epigenetic as well as structural roles (18, 19, 20). Finally, in order to clarify the mechanism of TTY2 genes in the process of spermatogenesis, bioinformatics approaches such as study of sequence similarity of this gene with known genome sequences and experimental approaches such as knockdown of TTY2 transcriptions by RNA interference technique or cytological study of testis in patients with deletions of these genes are recommended.

## References

1. Shabanipour S, Mashayekhi F, Bahadori MH, Soruri ZZ. The relationship between MMP-9 promoter polymorphism and IVF outcome. *Cell. Mol. Biol.* 2015; 61:64-7.
2. Zare-Abdollahi D, Safari S, Mirfakhraie R, Movafagh A, Bastami M, Azimzadeh P, Salsabili N, Ebrahimizadeh W, Salami S, Omrani MD. Mutational screening of the NR5A1 in azoospermia. *Andrologia.* 2015; 47:395-401.
3. Rai V. Genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) gene and susceptibility to depression in Asian population: a systematic meta-analysis. *Cell. Mol. Biol. (Noisy-le-grand).* 2014; 60 :29-36.
4. Novin MG, Mirfakhraie R, Nazarian H. Aberrant Wnt/ $\beta$ -Catenin Signaling Pathway in Testis of Azoospermic Men. *Advanced pharmaceutical bulletin.* 2015; 5:373-80.
5. Nazarian H, Novin MG, Jalili MR, Mirfakhraie R, Heidari MH, Hosseini SJ, Norouzian M, Ehsani N. Expression of Glycogen synthase kinase 3- $\beta$  (GSK3- $\beta$ ) gene in azoospermic men. *Iranian journal of reproductive medicine.* 2014; 12:313-19.
6. Mahat RK, Arora M, Bhale DV, Holkar S, Kumar S, Yadav T. Risk Factors and Causes of Male Infertility-A Review. *Biochemistry & Analytical Biochemistry.* 2016; 5: 271-8.

7. Ansari-Pour N, Razaghi-Moghadam Z, Barneh F, Jafari M. Testis-Specific Y-Centric Protein-Protein Interaction Network Provides Clues to the Etiology of Severe Spermatogenic Failure. *Journal of proteome research*. 2016; 15:1011-22.
8. Song SH, Chiba K, Ramasamy R, Lamb DJ. Recent advances in the genetics of testicular failure. *Asian J Androl* 2016;18:350-5
9. Hu YC, Namekawa SH. Functional significance of the sex chromosomes during spermatogenesis. *Reproduction*. 2015; 149: 265-77.
10. Zhu Y, Wu T, Li G, Yin B, Liu H, Wan C, Zhang H, Zeng Y. The sperm quality and clinical outcomes were not affected by sY152 deletion in Y chromosome for oligozoospermia or azoospermia men after ICSI treatment. *Gene*. 2015; 573:233-8.
11. Ambasudhan R, Singh K, Agarwal JK, Singh SK, Khanna A, Sah RK, Singh I, Raman R. Idiopathic cases of male infertility from a region in India show low incidence of Y-chromosome microdeletion. *Journal of biosciences*. 2003; 28:605-12.
12. Ceylan GG, Ceylan C. Genetics and male infertility. *World J Clin Urol*. 2015; 4:38-47.
13. Skakkebaek NE, Rajpert-De Meyts E, Louis GM, Toppari J, Andersson AM, Eisenberg ML, Jensen TK, Jørgensen N, Swan SH, Sapra KJ, Ziebe S. Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*. 2016; 96:55-97.
14. Koh E. PL 36. A novel Y chromosome microdeletion with the loss of an endogenous retrovirus related, testis specific transcript in AZFb region. *Translational Andrology and Urology*. 2012; 1.
15. Makrinou E, Fox M, Lovett M, Haworth K, Cameron JM, Taylor K, Edwards YH. Tty2: a multicopy y-linked gene family. *Genome research*. 2001; 11:935-45.
16. Yapijakis C, Serefoglou Z, Papadimitriou K, Makrinou E. High frequency of TTTY2-like gene-related deletions in patients with idiopathic oligozoospermia and azoospermia. *Andrologia*. 2015; 47:536-44.
17. Ghorbian S. Routine diagnostic testing of Y chromosome deletions in male infertile and subfertile. *Gene*. 2012; 503:160-4.
18. Wu H, Sun L, Wen Y, Liu Y, Yu J, Mao F, Wang Y, Tong C, Guo X, Hu Z, Sha J. Major spliceosome defects cause male infertility and are associated with nonobstructive azoospermia in humans. *Proceedings of the National Academy of Sciences*. 2016; 113:4134-9.
19. Li Z, Huang Y, Li H, Hu J, Liu X, Jiang T, Sun G, Tang A, Sun X, Qian W, Zeng Y. Excess of rare variants in genes that are key epigenetic regulators of spermatogenesis in the patients with non-obstructive azoospermia. *Sci Rep*. 2015;5:8785.
20. Yao C, Liu Y, Sun M, Niu M, Yuan Q, Hai Y, Guo Y, Chen Z, Hou J, Liu Y, He Z. MicroRNAs and DNA methylation as epigenetic regulators of mitosis, meiosis and spermiogenesis. *Reproduction*. 2015; 150: 25-34.