The Natural History of a Man With Ovotesticular 46,XX DSD Caused by a Novel 3-Mb 15q26.2 Deletion Containing *NR2F2* Gene

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Gonadal sex determination is a complex genetic process by which an embryonic primordium is driven to form an ovary or a testis, which requires a delicate dosage balance involving many genes. Disruption in this molecular pathway can lead to differences of sex development (DSD). Although some genetic mechanisms leading to 46,XY DSD have been elucidated, little is known about copy-number variation (CNV) causing testicular or ovotesticular 46,XX DSD. We describe a 20-year natural history of a man with *SRY*-negative 46,XX who was born with atypical male external genitalia, aortic coarctation, and bilateral blepharophimosis-ptosis. The molecular study identified a *de novo* heterozygous 3-Mb 15q26.2 deletion, a gene-poor *locus* containing *NR2F2*, which encodes the nuclear receptor COUP-TFII that is highly expressed in ovary and cardiac arteries. Immunohistochemistry confirmed the low COUP-TFII expression on his ovotestis tissue. Monosomy of 15q26.2, encompassing the *NR2F2* gene, may act as a Z-factor regulating the male sex determination negatively. This finding supports a novel type of CNV resulting in DSD in an individual who developed male puberty spontaneously.

Freeform/Key Words: 15q26.2 deletion, differences of sex development, gene regulation, copy-number variation, transcription factor binding sites, 46,XX DSD

46,XX ovotesticular differences/disorders of sex development (DSD), formerly known as true hermaphroditism [1], comprise a spectrum of sex anatomy promoted by rare variants of sexually dimorphic gonadal genes [2]. The sex determination in humans is a complex

Abbreviations: CNV, copy-number variation; DSD, differences of sex development; TFB, transcription factor binding.

Received 22 June 2019
Accepted 22 August 2019
First Published Online 28 August 2019

ISSN 2472-1972
November 2019 | Vol. 3, Iss. 11
doi: 10.1210/js.2019-00241 | Journal of the Endocrine Society | 2107–2113

signaling pathway, which requires a delicate dosage balance involving many genes, acting either synergistically or antagonistically [3]. Disruption of these molecular pathways can lead to DSD. Although some genetic mechanisms leading to 46,XY DSD have been elucidated, little is known about testicular or ovotesticular 46,XX DSD [2, 3]. Approximately 80% of all 46,XX testicular DSD cases are explained by the presence of the *SRY* gene [1]. Other rare gain-of-function mechanisms include duplications of *SOX9* or its regulatory region [2], copy-number variations (CNV) disrupting the regulatory region *SOX3* [4], and partial duplications of human chromosome 22q13, containing *SOX10* [2]. Additionally, loss-of-function mutations in female sex-determining (putative pro-ovary) genes can also lead to 46,XX DSD. It has been described in mutations in *RSPO1* and *WNT4*, causing testicular 46,XX DSD. Mutations in *RSPO1* are associated with palmoplantar hyperkeratosis and the susceptibility to squamous cell carcinoma [2, 5]. Also, identical *NR5A1* mutations have been found in two unrelated patients with testicular/ovotesticular 46,XX DSD [6]. More recently, heterozygous mutations in *NR2F2* have been linked to testicular/ovotesticular 46,XX DSD in children with cardiac defects [7].

Although the above genetic events are associated with 46,XX males, there is still much to be learned for the full understanding of ovotesticular DSD. In addition, incomplete penetrance and intrafamilial phenotype variability have been described among DSD-gene mutation carriers [8]. Notwithstanding, some recognized chromosomal rearrangements had been associated with urogenital and gonadal phenotypes, two being located at 15q21 and 15q24.1. Herein, we describe the natural history of the entire puberty development of a 46,XX ovotesticular DSD man caused by a novel 3-Mb 15q26.2 deletion, containing the *NR2F2* gene. This region may function as the Z-factor [9], in which it plays a fundamental role in ovary development, acting as a negative regulator of male sex determination.

1. Case Report

A. Clinical History

The reported patient is a 20-year-old man diagnosed with ovotestis. He was born from nonconsanguineous parents after an uneventful pregnancy, the third youngest boy in a healthy family. His lymphocyte culture revealed a 46,XX karyotype. At birth, a 3-cm penis with midshaft hypospadia, palpated inguinal gonads, and bilateral blepharophimosis-ptosis were detected. Low-set ears, webbed neck, pectus excavatum, and sinus bradycardia were noted (Fig. 1A and 1B). He presented low weight (5th percentile) but standard stature (75th percentile) for his age and surpassed parental target height (Fig. 1C). Asymmetric blepharophimosis-ptosis was documented by his parents (Fig. 1D). Urethrography showed a small prostatic utricle and a failure to fill the contrast in the posterior urethra (Fig. 1E). Cystoscopy identified a prominent bulging corresponding to verumontanum (Fig. 1F). Corneal topography diagnosed keratoconus when he was 11 years old (Fig. 1G). He underwent zetaplasty when he was 12 years old for correcting penile curvature that developed late after hypospadia repairs (Fig. 1H). At ~14 years of age, he began spontaneous puberty, even though he presented with bilateral microorchidism (1 and 1.8 mL). He attained pubertal spurt and male secondary characteristics and no gynecomastia was evidenced. He developed an average penile length, male muscular distribution, and pubic hair, although scarce on the face (Fig. 1A). When he was 16 years old he underwent left gonadal biopsy and right gonadectomy because of a firm nodule in the right gonad diagnosed on histopathology as fibrotic tissue. Revised gonadal biopsies confirmed ovotesticular 46,XX DSD, comprising follicular epithelium mainly with granulosa cells in the ovarian part and Leydig cell hyperplasia and Sertoli cell-only tubules in the testicular part (Fig. 2A–2D). Gonadal axis tests are summarized in Table 1. He was assigned and raised as a boy, identified himself as male during childhood and adolescence, and has been living as heterosexual.

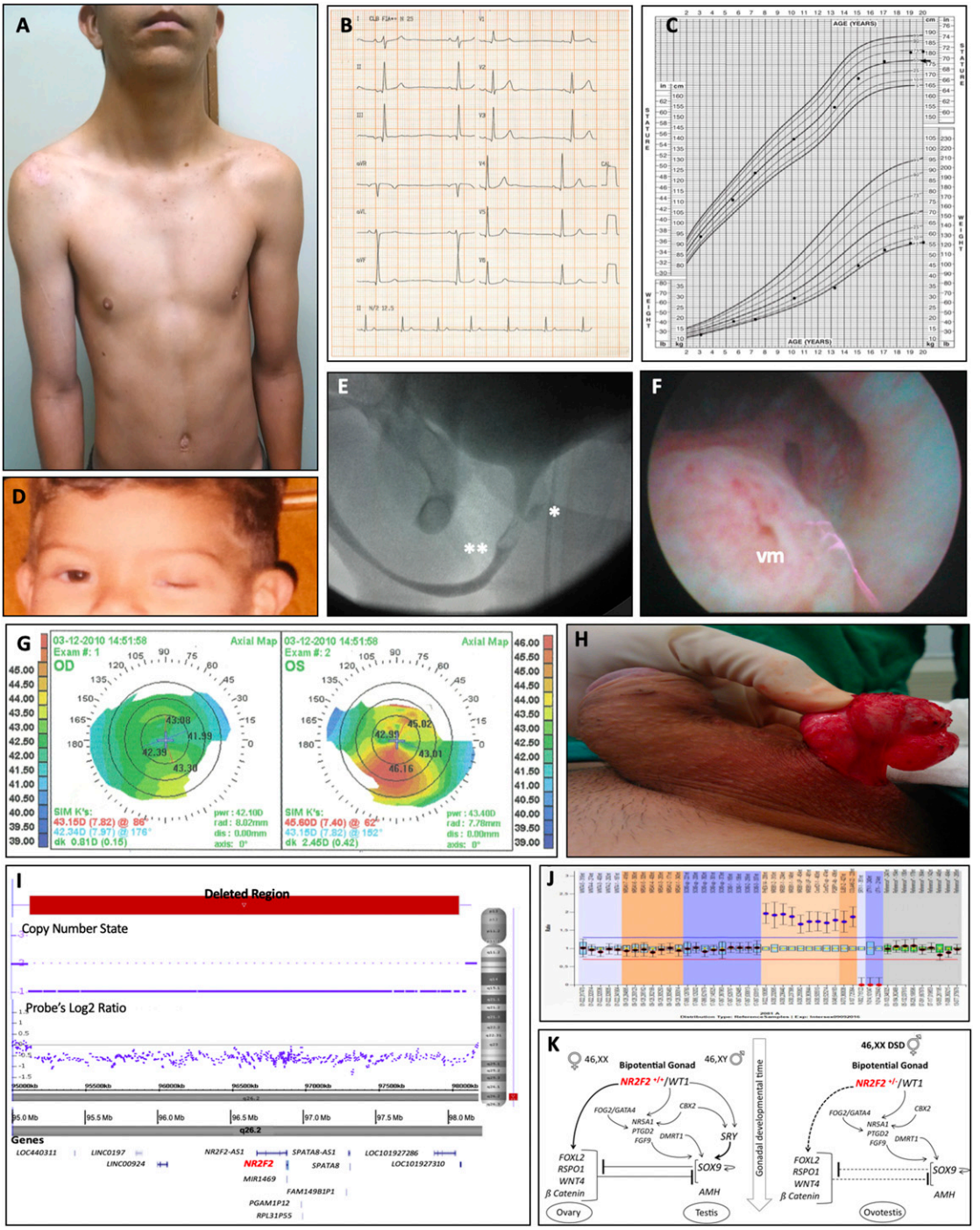

Figure 1. Clinical and surgical investigation of a 46,XX man carrying heterozygous deletion at 15q26.2 encompassing *NR2F2* gene. (A) Low-set posteriorly rotated ears, webbing of the neck, and mild pectus carinatum inferiorly with pectus excavatum superiorly. (B) Holter monitor indicating sinus bradycardia with the average measurement of 48 bpm. (C) Growth chart during clinical and surgical follow-up indicating his height (in/cm) and weight (ib/kg). (D) Asymmetric blepharophimosis-ptosis when he was 2 y old. (E) Urethrography showing the presence of a small prostatic utricle (*) and a failure to fill the contrast in the posterior urethra (**). (F) Cystoscopy identified a prominent lesion in verumontanum (vm) corresponding to the same position as the contrast-fill failure in urethrography. (G) Corneal topography indicating keratoconus when he was 11 y old during an investigation for low visual acuity. (H) Intraoperative macroscopic aspect of the right gonad obtained during zetaplasty performed for the treatment of penile curvature when he was 12 y old, which emerged in late postoperative of hypospadia repairs at 4 and 6 y old, showing

a cystic structure close to the gonad that was identified as Müllerian remnant. (I) Chromosomal microarray result showing a *de novo* 3-Mb 15q26 deleted segment (red bar) using UCSC hg19 genome reference (horizontal gray bar) placed in a gene-poor region (transcripts; dark blue) including *NR2F2* gene (red). (J) SALSA MLPA P185 Intersex® depicting patient's probing for *NR0B1* (*DAX1*) and *CXorf21* on Xp21.2, *SOX9* on 17q24.3, *SRY* and *ZFY* on Yp11.3, *WNT4* on 1p36.12 and *NR5A1* on 9q33, in addition to specific probes for the X- and Y-chromosomes. (K) Proposed NR2F2 signaling pathway for ovary and testis development. In XX embryos, this pathway occurs whether *NR2F2* is in two functional copies. When carrying *NR2F2* heterozygous ($^{+/-}$) deletion, the upregulation of putative pro-ovary genes is diminished. Although in the absence of *SRY*, *SOX9* is activated, but not sufficiently expressed to downregulate pro-ovary genes. In this peculiar context, neither pro-ovary genes are strong enough to inhibit the putative protestis *SOX9*, neither *SOX9* is enough to inhibit pro-ovary genes, therefore, leading to ovotestis development.

B. Genetic and Immunohistochemistry Analyses

Upon detailing his 46,XX karyotype, single nucleotide polymorphism-array analysis was performed and revealed a *de novo* 3-Mb deletion on 15q26: arr[GRCh37]15q26.2(95127653_98146649)×1, causing partial 15q monosomy (Fig. 1I). This region is an evolutionarily conserved *locus* among mammals and contains two genes (*NR2F2* and *SPATA8*), three noncoding genes (*NR2F2-AS1*, *SPATA8-AS1*, and miR-1469), and three pseudogenes (*PGAM1P12*, *RPL31P5*, and *FAM149B1P1*) (Fig. 1I). The *locus* 15q26.2 also encompasses regulatory regions, containing more than 15,000 transcription factor binding (TFB) sites, among them, 282 to SRY, 147 to SOX5 and 103 to SOX9 (data not shown). PCR and Sanger sequencing from peripheral blood DNA were negative for common 46,XX DSD variants in *SRY*, *RSPO1*, *SOX9*, as well as *NR2F2*. MLPA revealed no CNV for *WNT4*, *NR5A1*, and *SOX9* (Fig. 1J). Additionally, whole-exome sequencing performed in the proband and his parents resulted in no pathogenic DSD variants.

Once we ruled out *SOX9*, *SOX3*, *WNT4*, *FOXL2*, *RSPO1*, and *NR2F2* missense mutations and that *NR2F2* is missing in the 3-Mb 15q26 deletion, we further performed immunohistochemistry in the resected gonadal tissue and found lower COUP-TFII expression in granulosa-type cells and Sertoli cells compared with sex-cord stromal cells (Fig. 2E–2F).

2. Discussion

A *de novo* 3-Mb deletion on 15q26, encompassing *NR2F2* gene and hundreds of sex-associated TFB sites, constitutes a novel genetic rearrangement in a 46,XX subject born with atypical male genitalia who developed all secondary male sexual characteristics. Examining his histopathology gonad thoroughly, we hypothesized that the ovotestis might have evolved to progressive gonadal fibrosis. Likely it may have begun slightly during the fetal period and then gradually increased up to the time of the midfinal period of puberty. The gradual testis atrophy leading to micro-orchidism must have resulted in Sertoli cells death, therefore, a decrease of inhibin B secretion and FSH increase as observed at the end of his adolescence. By compromising Leydig cells subsequently, the ongoing atrophy might have resulted in decreased testosterone synthesis and elevated LH level by a negative feedback loop.

This new 15q26.2 CNV implies NR2F2/COUP-TFII haploinsufficiency and is consistent with its diminished expression observed in ovotestis gonad, indicating its role in controlling DSD. Many recognized chromosomal rearrangements had been associated with urogenital and gonadal phenotypes [8]. In 2017, we reported the 15q26.2 CNV in association with 46,XX ovotesticular DSD, and cardiac phenotype [10]. Most CNVs are related to a loss-of-function mechanism, and few are related to upregulation of putative protestis factors in the developing 46,XX gonads as reported in *SOX9* and *SOX3* [8]. Other 46,XX testicular or ovotesticular DSDs result from mutations in pro-ovary/antitestis genes of the *WNT4*/*RSPO1* signaling pathway, including loss-of-function mutations in *WNT4* and homozygous *RSPO1* mutations. Also, mutations in the *FOXL2* gene are linked to ovary insufficiency and blepharophimosis-ptosis phenotype [8].

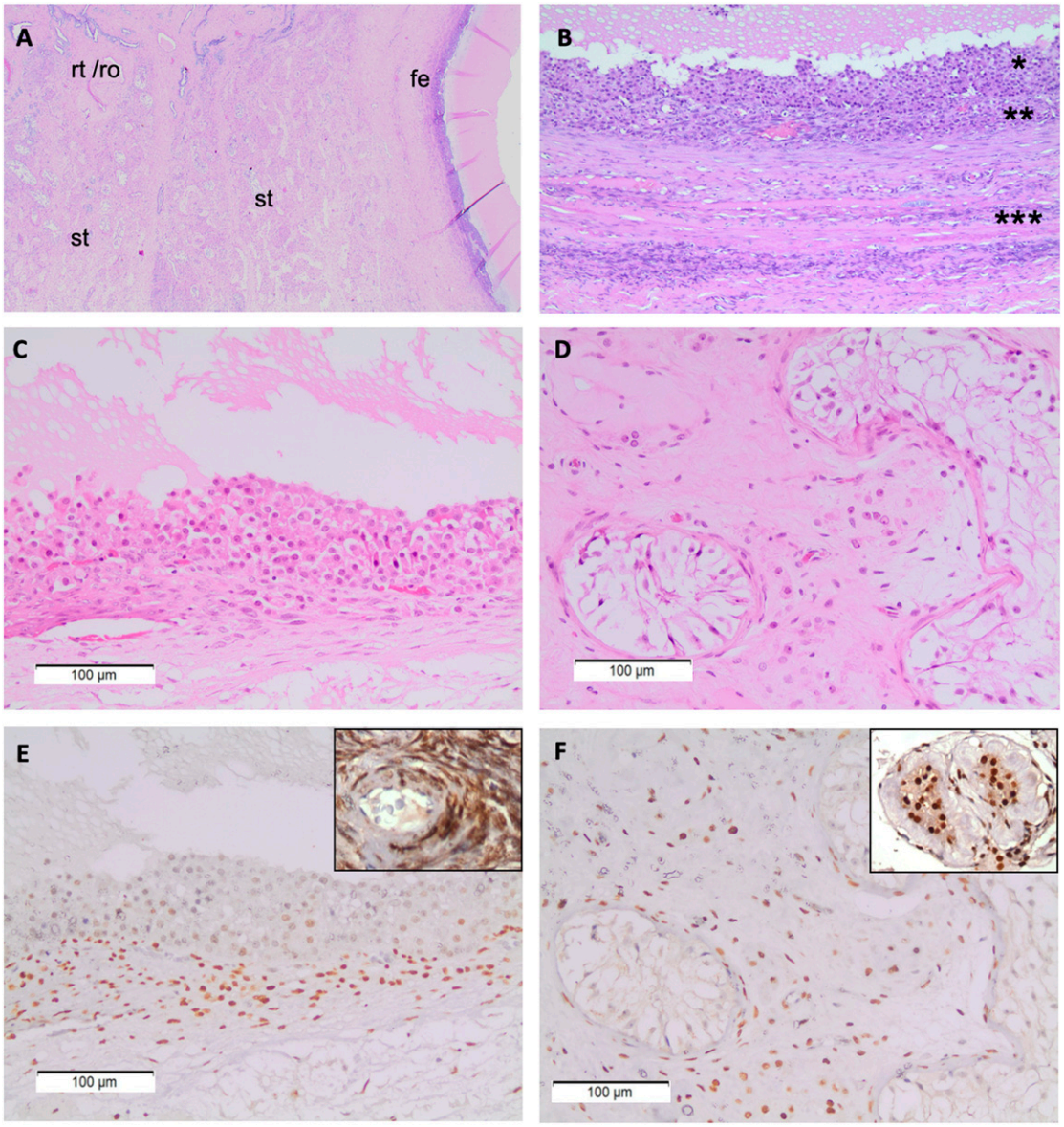

Figure 2. Immunohistochemical characterization of steroid hormone nuclear receptor COUP-TFII expression on the ovotestis gonad from 46,XX subject carrying a 3-Mb 15q26.2 heterozygous deletion. (A) Low-power scanning field showing left to right ovotesticular dysgenetic gonad containing testicular seminiferous tubules (st), merged rete testis/rete ovarii (rt/ro), and ovarian follicular epithelium (fe) with hematoxylin and eosin (H&E) staining. (B) Dysgenetic ovarian follicular epithelium showing granulosa (*) and theca (**) cell layers, surrounded by ovarian stroma (***) on H&E (200×). (C) Granulosa cells lining in a theca lutein cyst where no primary oocytes were found (H&E). (D) Seminiferous tubules are devoid from germinative cells and contain only Sertoli cells surrounded by some luteinized stromal cells (H&E). Weak nuclear immunostaining for COUP-TFII immunoperoxidase in granulosa (E) and Sertoli (F) cells fulfilling seminiferous tubules of ovotesticular tissue in comparison with ovary (from young woman/ 200×) and testis (elderly man / 400×) external control tissues used in the same immunostaining bath, which are zoomed in the upper-right-hand corner of E and F.

The verified *NR2F2* heterozygous deletion can explain the genital ridge mesenchyme switch from an ovary to testis caused by the disruption of ovary-specific signaling, which would oppose testis differentiation. Zhao *et al.* (2017) recently showed that *Nr2f2* ($^{-/-}$) 46,XX mouse embryos developed mixed ductal mesenchyme, leading to both female and male reproductive tracts [11]. Our report is in agreement with Zhao's active pro-ovarian mechanism and validates in human the

Table 1. Gonadal Axis Hormone Measurements During 20-Year Follow-Up of a Man with Ovotesticular 46,XX Due to 15q26.2 Haploinsufficiency

Period	Age (yr)	Total T ng/dL (male NR)	LH IU/L (NR)	FSH IU/L (NR)
Newborn	66 d	120 (<30)	NA	NA
During childhood	2	30 (<30)	0.52 (<0.10)	3.0 (<0.3)
	3.2	22 (<30)	0.90 (< 0.10)	2.2 (<0.3)
Very early to late adolescence	11.7	127 (30–150)	NA	NA
	12.5	188 (30 –150)	1.86 (<2.28)	4.6 (0.30 –4.00)
	13	202 (30–150)	5.1 (0.31–5.29)	6.9 (0.30 –4.00)
	14.6	340 (241–827)	4.8 (0.31–5.29)	19.1 (0.40–7.40)
	16	407 (241–827)	32.4 (1.50–9.30)	63.8 (0.40–7.40)
	17.8	177 (241–827)	39 (1.50 –9.30)	91 (0.40–7.40)
	19.7	122 (241– 827)	43 (1.50 –9.30)	97.7 (0.40–7.40)

Normal range refers to the interval of basal levels in control subjects matched according to age and male sex. The conversion factor used: testosterone ng/dL × 0.034 for nmol/L.
Abbreviations: NA, not available; NR, normal reference; T, testosterone.

hypothesis that the regression of Wolffian ducts in 46,XX embryos is actively driven by COUP-TFII by suppressing mesenchyme-epithelium crosstalk responsible for Wolffian maintenance [11]. It has been described that *NR2F2* is expressed at the same time of *WT1* in early gonadal embryogenesis. Therefore, our data are consistent with previous observations on the role of *NF2R2* [7, 11]. We hypothesized that the dosage-sensitive loss of *NR2F2* could release *WT1* to trigger the expression of *SOX9* through *NR5A1* in the absence of *SRY* (Fig. 2K).

Moreover, the 3-Mb 15q26.2 deletion also containing putative protestis (SOX9, SOX3, and SOX10) TFB sites may impair sex dimorphic regulation of target genes in the bipotential gonad. This regulatory haploinsufficiency can lead to incomplete testicular development, as observed in our patient, once SOX9 partial activation is not sufficient to completely block alleged pro-ovary gene expression (Fig. 2K). Bashamboo *et al.* [7], applying exome sequencing on 46,XX *SRY*-negative individuals with unexplained virilization or testicular/ovotesticular DSD, identified heterozygous mutations in *NR2F2* in three young children. They presented congenital heart disease, without palpable gonads, and two of them having blepharophimosis-ptosis-epicanthus inversus syndrome [7]. For that reason, the 15q26.2 region encompassing *NR2F2* and hundreds of putative protestis TFB sites may function as a Z-factor region, being crucial for ovary development, as hypothesized in 1993 by McElreavey *et al.* [9].

Therefore, our data show that the *locus* 15q26.2 can be considered one of the Z-factor regions for the sex development in XX embryos because both the monosomy of this chromosomal region and the haploinsufficiency of *NR2F2* are not capable of defining the full differentiation of either female or male gonads.

Acknowledgments

We thank Ilda Kunii for laboratory technical assistance, Professor Susan Lindsey for manuscript revision, and Professor Rui Maciel for sharing laboratory support.

Financial Support: This study was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants 2011/20747-8, 2012/00079-3, 2014/06570-6 and 2014/11572-8), CAPES and CNPq.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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