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Implication of *INSL3*, *INSL4* and *INSL6* in Male Fertility Development

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Letter to the Editor

The human insulin family members include; insulin-like growth factors I and II (*IGF-I* and *IGFII*); relaxins (*RLN1* and *RLN2*); Leydig cell insulin-like peptide (*INSL3*); early placenta insulin-like peptide (*INSL4*); and three recently discovered insulin-like hormones, *INSL5*, *INSL6*, and *RLN3* [1] as well as four relaxin/insulin like family peptide receptors (Table 1).

INSL3 is a considered to be a key hormone expressed shortly after sex determination and is involved in the regulation of the transabdominal phase of epididymo-testicular descent [2]. Of interest, *Insl3* mutant mouse epididymis lacks smooth musculature because of defective α-smooth muscle actin, which results in a high intraabdominal undescended position of the epididymo-testicular unit [3]. Emmen et al. reported that *Insl3* is not essential for Wolffian duct growth [4]. Therefore, only a combination of normal testosterone and *INSL3* secretion results in complete transabdominal descent of epididymo-testicular unit [5]. Furthermore, observed significant decline in *INSL3* gene signaling after GnRH treatment contrast to statement by Ivell at al. [6] that *INSL3* is not acutely regulated by the hypothalamic-pituitary-gonadal axis (Table 1).

INSL4 is known to be expressed in the human placenta [1]. Nothing is known about roles for *INSL4* and *INSL5* in male reproduction and only very little about relaxin-3, which is mostly considered as a brain peptide. Another member of insulin family, *INSL6* is highly expressed in post-meiotic germ cells. *Insl6* mutant mice show a marked disruption of spermatogenesis with meiotic arrest [7]. To date, however, no specific receptor for human *INSL6* has been discovered.

We selected 15 patients with isolated cryptorchidism, based on histological results, and divided them into 2 groups. Seven belonged to the Ad– (lacking Ad spermatogonia) and 8 to the Ad+ (presenting Ad spermatogonia) group. The patients had a median age of 18.5 months (range 8–59 months). Data from Ad– bilateral cryptorchid boys treated with GnRHa (Buserelin) following the first orchidopexy (surgery) (4 patients) were retrieved from randomized study [8]. Initial biopsies revealed no Ad spermatogonia, indicating defective mini-puberty (Ad– group). The second testis was managed by orchidopexy and biopsied 6 months after the initial surgery. Thus, results from 21 biopsies were compared. Patients were age and ethnicity matched. RNA sequencing data from two previous studies were used to analyze manually selected INSL family genes [9,10]. The histological analysis of biopsies, workflow from RNA isolation, through to purification, library preparation, sequencing, data analyses, and expression level analysis, has been previously described in detail [9,10]. Determination of differentially expressed genes, statistical analyses and model design were described previously [9,10]. Only genes with at least one read per million, in at least two samples, were included. P values and fold-changes were calculated for the treatment factor and differentially expressed genes were defined as those displaying a false discovery rate (FDR) of less than 0.05. Raw data files are available at the Database of Genotypes and Phenotypes (dbGaP) with the accession number phs001275.v1.p1.

Long term follow-up studies showed that because of impaired mini-puberty, 97% of cryptorchid HIR males were infertile with an average of 9.1 × 10.6 sperm per ejaculate, while 33% out of this group developed azoospermia [11]. If cryptorchid boys with impaired mini-puberty received treatment with a GnRH analogue following orchidopexy, a normal sperm count was achieved in 86% of subjects [12]. Thus, hormonal treatment with GnRHa in early childhood permanently restored fertility and prevented the development of azoospermia [12].

Testes with defective mini-puberty, with lower testosterone levels, and lack of Ad spermatogonia had significant lower RNA levels *INSL6* relative to testes with Ad spermatogonia (Table 1). It is possible therefore that differentially expressed *INSL6* gene reflect molecular functions involved in the gonocyte-to-Ad spermatogonia transition in humans during mini-puberty.

Surprisingly, GnRHa treatment did not induce increased expression of *INSL6* although it induced Ad spermatogonia differentiation [12]. Observed alternative pathway activation with upregulation of *INSL4* indicates a novel role for this gene. Thus, *INSL4* and *RXFP1* as well as *RXFP2* indicate new contributors for Ad spermatogonia differentiation and male fertility development. Of notice, *IGF1* expression was downregulated after GnRH treatment supporting the idea that GnRHa induce primarily differentiation and not a self-renewal of spermatogonia.

Conclusion

In conclusion, *EGR4* and *PITX1* controlled by *PROK2/CHD7/FGFR1/SPRY4* genes are responsible for LH deficiency, which in turn affects the germ cell transitional effectors, *FGFR3, FGF9, NANOS2, NANOS3, SOHLH1* and *SOHLH2* [13,14]. Upon GnRHa treatment, however, alternative pathways are activated, including the LH-secretion orchestrating factors, *EGR2, EGR3, TAC1, TAC3, PROP1* and *LEP*, as well as the gonocyte-to-Ad spermatogonia transition effectors, *DMRTC2, T, PAX7, TERT, NRG1, NRG3, RBMY1B, RBMY1E* and *RBMY1J* [13,14] and newly, *INSL4*, *RXFP1* and *RXFP2*.

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Table 1: Differential expression of INSL family genes in Ad- (HIR) versus Ad+ (LIR) group and in the GnRHa treated versus untreated group (GnRHa).

Gene ID	Name	logFC HIR/LIR	FDR HIR/LIR	logFC GnRHa	FDR GnRHa
INSL1/IGF1	insulin-like 1	n.s..	n.d.	-1.51	9.5E-08
INSL2/IGF2	insulin-like 2	n.s..	n.s.	-n.s.	n.s.
INSL3	insulin-like 3	n.s.	n.s..	-1.01	0.003
INSL4	insulin-like 4	n.d.	n.d.	2.31	0.007
INSL5	insulin-like 5	n.d.	n.d.	n.d.	n.d.
INSL6	insulin-like 6	-2.06	0.006	n.d.	n.d.
RFXP1	relaxin/insulin-like family peptide receptor 1	n.s.	n.s.	1.06	0.002
RFXP2	relaxin/insulin-like family peptide receptor 2	n.d.	n.d.	1.38	0.046
RFXP3	relaxin/insulin-like family peptide receptor 3	n.d.	n.d	n.d.	n.d.
RFXP4	relaxin/insulin-like family peptide receptor 4	n.d.	n.d.	n.d	n.d
RLN1	relaxin 1	n.d.	n.d.	n.d.	n.d.
RLN2	relaxin 2	n.d.	n.d.	n.d.	n.d.
RLN3	relaxin 3	n.d.	n.d.	n.d.	n.d.

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