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GnRHa Treatment of Cryptorchid Boys Affects Genes Involved in Hormonal Control of the HPG Axis and Fertility

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Keywords

Cryptorchidism · GnRHa treatment · Infertility · RNA sequencing

Abstract

The gonadotropin-releasing hormone agonist (GnRHa; Buserelin) rescues fertility during adulthood in the majority of high infertility risk cryptorchid boys presenting with defective mini-puberty. However, the molecular events governing this effect are not understood. We report the outcome of an RNA profiling analysis of testicular biopsies from 4 operated patients who were treated with GnRHa for 6 months versus 3 operated controls who were not treated. GnRHa induces a significant transcriptional response, including protein-coding genes involved in pituitary development, the hypothalamic-pituitary-gonadal axis, and testosterone synthesis. Furthermore, we observed an increased abundance of long noncoding RNAs (lncRNAs) participating in epigenetic processes, including *AIRN*, *FENDRR*, *XIST*, and *HOTAIR*. These data are consistent with the hypothesis that hypogonadotropic hypogonadism in boys with altered mini-puberty is the consequence of a profoundly altered gene expression program involving protein-coding genes and lncRNAs. Our results point to molecular mechanisms that underlie the ability of GnRHa to rescue fertility.

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Early treatment of cryptorchidism to improve fertility is the recommended course of action [Ritzén et al., 2007; Kolon et al., 2014]. However, not all cryptorchid boys will develop into fertile adults in spite of timely corrective surgery [Hadziselimovic and Herzog, 2001]. At the molecular level, numerous differences in gene expression between high infertility risk (HIR) and low infertility risk (LIR) groups were observed, which underscores the importance of an intact hypothalamic-pituitary-gonadal (HPG) axis for the development of normal adult spermatogenesis [Hadziselimovic et al., 2009,

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2011; Chalmel et al., 2012]. These data strongly support the theory that in HIR cryptorchid boys, insufficient *PROK2*, *CHD7*, *FGFR1*, and *SPRY4* gene expression induces deficient LH secretion, which results in impaired mini-puberty and ultimately in adult infertility [Hadziselimovic et al., 2016]. In male infants, gonadotropin secretion increases between 2 and 4 months after birth, which stimulates Leydig cells to secrete testosterone [Forest et al., 1974; Winter et al., 1976]. This testosterone increase is blunted in cryptorchid infants [Gendrel et al., 1978], which results in an inadequate transition of gonocytes into A dark (Ad) spermatogonia [Bica and Hadziselimovic, 1992; Zivkovic et al., 2007]. Consequently, establishing adult fertility following successful surgery is largely dependent on a normal mini-puberty and, as a result, the presence of Ad spermatogonia in the testis [Hadziselimovic et al., 2005; Hadziselimovic and Hoecht, 2008]. In a prospective study, it was found that 47% of cryptorchid patients undergoing surgery lacked Ad spermatogonia in their testis and where thus highly likely to become infertile as adults [Bilius et al., 2015]. In 1997, cryptorchid boys of at least 8 years of age, who were treated with gonadotropin-releasing hormone agonist (GnRHa; Buserelin), showed improved sperm concentrations when compared to an untreated control group [Hadziselimovic and Herzog, 1997]. GnRHa treatment on alternate days over a period of 6 months did not inhibit gonadotropin secretion, while levels of LH increased and atrophic juvenile Leydig cells regenerated towards the end of the treatment [Hadziselimovic et al., 1984]. Thus, the treatment increased the number of germ and Leydig cells [Hadziselimovic et al., 1984, 1987]. Importantly, long term follow-up in HIR cryptorchid boys treated before the age of 6 showed normal sperm concentrations for 86% of the cases [Hadziselimovic, 2008].

This result showed for the first time that infertility resulting from undescended testis, which was believed to be an incurable congenital malformation, could be prevented. However, little is known about the molecular mechanisms governing this phenomenon, and no large-scale expression data are available on the short-term effects elicited by a 6-month hormonal treatment for HIR patients as compared to surgery alone. This study presents RNA profiling data that reveal the transcriptional effects elicited by GnRHa treatment. Critically, we identified genes that regulate the pituitary testicular axis development in both the canonical and alternate pathways, as well as long noncoding RNAs (lncRNAs), including some that are involved in epigenetic processes.

Subjects and Methods

Study Population and Biopsy Sample Collection

Seven bilateral cryptorchid boys with a median age of 18.5 months (range 8–59 months), who had received no hormonal or surgical treatment prior to randomization, were selected. Data from patients treated either with surgery and GnRHa (4 patients) or with surgery alone (3 patients) were retrieved from an ongoing randomized study for further analysis. We included 14 biopsies of 7 patients before and after the treatment. Initial biopsies revealed no Ad spermatogonia, indicating a defective mini-puberty. The second testis of all patients was orchidopexied and biopsied 6 months after their initial surgery. In addition, RNA sequencing data from our previous study (7 HIR and 8 LIR patients) were included [Hadziselimovic et al., 2016]. Thus, altogether results from 22 patients were compared. Boys in the GnRHa-treated group received 10 μg GnRHa (Buserelin), administered as a nasal spray, in the evening of every second day over a period of 6 months. A cryptorchid testis is defined as a testis localized outside of the scrotum and incapable of being brought into a stable scrotal position. All undescended testes in this study were located in the inguinal region. The cryptorchid boys entering the study underwent an extensive examination with no clinical signs of developmental malformations or syndromes. Performing clinical examination in accordance with STROBE criteria for case-controlled studies [von Elm et al., 2014], we excluded patients with small testes, a small penis, or a lack of normal scrotal rugae, pigmentation, and gynecomastia. We further determined serum FSH, LH, testosterone, and inhibin levels [Verkauskas et al., 2016]. No MRI scans of the brain and sella turcica were performed. No clinical symptoms were found for hyperprolactinemia, pituitary lesions (tumor, granuloma, and abscess), Cushing syndrome, severe or chronic illness, trauma or surgery, or genetic mutations such as Prader-Willi syndrome. None of our patients suffered from a systemic disease such as hemochromatosis, sarcoidosis, or histiocytosis X. Testicular biopsies were taken at the time of orchidopexy. A 5-mm incision was made into the tunica albuginea in the superior pole of the testis, and a sample of approximately the size of a rice grain was isolated from the protruding tissue using separate blades. This sample was then subdivided, with one fragment fixed in glutaraldehyde for histological processing, while the other one was immediately immersed in RNAlater (ThermoFisher Scientific) and stored at −25 °C until further processing (for RNA extraction and RNA sequencing).

Histological Analyses

Biopsies were fixed in 3% glutaraldehyde in PBS and then embedded in Epon resin. Semi-thin sections (1 μm) were cut using a Reichert Om-U3 ultramicrotome. Sections were mounted on glass slides, stained with 1% toluidine blue, and examined under a light microscope at a total magnification of ×600. Biopsies were histologically examined by 2 of the authors (FH and DD), each with expertise in the interpretation of semi-thin sections of prepubertal testes.

During histological analyses, at least 100 tubular cross sections per biopsy were evaluated with regard to their number and absence of Ad spermatogonia. In the prepubertal testis, Ad spermatogonia were identified according to the criteria first published by Seguchi and Hadziselimovic [1974]. This type of germ cell has a typical halo in the nucleus, termed the rarefication zone, and a cytoplasm with

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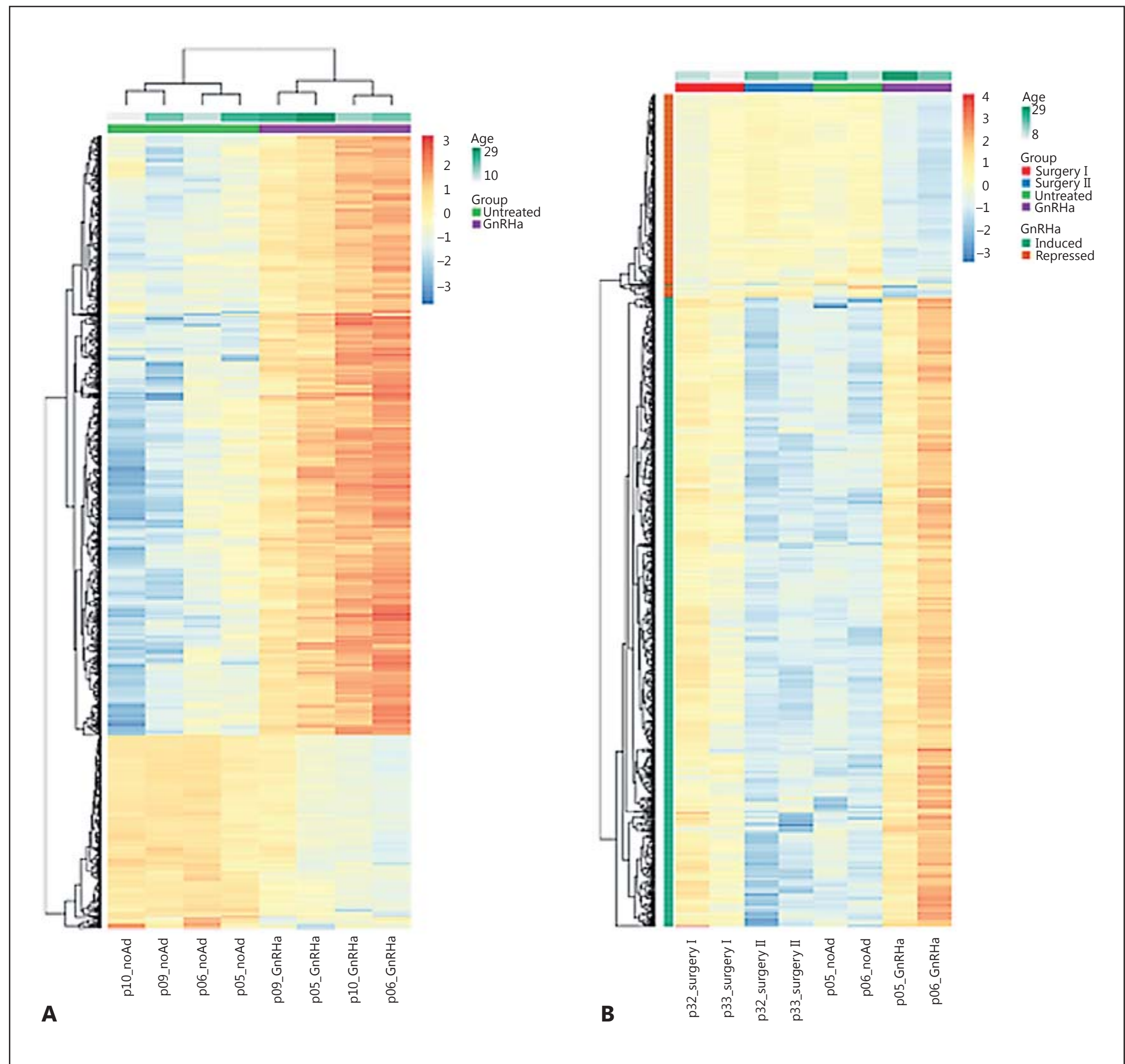

Fig. 1. Heatmap of differential expressed genes showing the log2 expression of the 1,000 most significant differential genes between before/after GnRHa treatment (**A**) and between surgery-only and GnRHa-treated patient groups (**B**). Patient group and age are color-coded and indicated on the top and on the right side of each heatmap. Genes have been reordered by hierarchical clustering to group similar profiles (clustering dendrograms shown on the left side).

a darker aspect in comparison to Ap or fetal spermatogonia. Crucially, the predominant factor in the development of infertility is the observation that gonadotropin levels show a more striking correlation with the presence or absence of Ad spermatogonia in both gonads than with the category of undescended testes, i.e., unilateral or bilateral [Hadziselimovic and Hoecht., 2008].

RNA Preparation, Sequencing, Data Analyses, and RNA Expression Levels

The workflow from RNA isolation to purification, library preparation, sequencing, data analyses, and expression level analysis has been described previously (HIR/LIR differential gene expression study) [Hadziselimovic et al., 2016]. These steps were performed in parallel in an identical fashion for the GnRHa-treated and surgery-only groups.

Data and Differential Gene Expression Analyses

To determine genes differentially expressed before and after GnRHa treatment, the counts per gene sample from patients treated with GnRHa were statistically analyzed in R using the edgeR package [Robinson et al., 2010] by fitting a quasi-likelihood negative binomial generalized log-linear model for each gene [Lund et al., 2012]. The model was designed with 2 additive categorical factors: (1) *patient*, which identifies measurements from the same patient, before and after GnRHa treatment, and controls for gene expression differences between individuals, and (2) *treat-*

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ment, which models gene expression changes induced by GnRHa. Only genes with at least 1 read per million in at least 2 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 <0.05 and an absolute change in expression of at least 2-fold. FDR-controlling procedures are designed to estimate the expected proportion of "discoveries" (rejected null hypotheses) that are false (incorrect rejections) and is one way of conceptualizing the rate of type I errors in null hypothesis testing when conducting multiple comparisons. FDR-controlling procedures provide less stringent control of type I errors compared to familywise error rate (FWER) controlling procedures (such as the Bonferroni correction), which control the probability of at least one type I error. Thus, FDR-controlling procedures have greater power at the cost of increased rates of type I errors. Cut-off was not applied to raw *p* values, but significant values of <0.05 were corrected for multiple testing; namely FDR was calculated after the Benjamini and Hochberg [1995] approach.

Protein Interaction Network

Genes involved in the HPG axis, pituitary and neuronal development, and differentially expressed in the HIR versus LIR group, together with the genes differentially expressed in the GnRHa-treated versus untreated group (146 genes in total) were used as inputs to obtain the protein-protein interaction network using STRING version 10.0 [Szklarczyk et al., 2015].

Results

GnRHa Treatment but Not Surgery Alone Affects Gene Expression

RNA profiling of samples from 4 patients before and after GnRHa treatment detected 28,645 transcripts. Of these, 6,469 showed a significant difference in concentration levels, whereby an increased expression was observed for 5,823 cases (90%) following GnRHa treatment (FDR <0.05; absolute expression change ≥2-fold). Distinct gene expression profiles before and after GnRHa treatment were identified and are summarized in Figure 1A (online suppl. Table 1; for all online suppl. material see www.karger.com/doi/10.1159/000471937).

Next, we asked whether a transcriptional response was triggered in patients who had undergone only orchidopexy without GnRHa treatment. A differential expression analysis that included paired samples from patients before/after surgery and with/without GnRHa treatment indicated that only very few significant effects were elicited at the transcriptional level following solely surgical intervention (Fig. 1B, compare lanes 1/3 and 2/4). This finding is in agreement with the common pathology found in biopsies from both testes and confirms previous observations that both testes in bilateral cryptorchidism have the same abnormal testicular histology and low numbers of spermatogonia [Hedinger, 1979].

GnRHa-Responsive Genes Act in Pathways Critical for the Hormonal Control of Sexual Reproduction, Pituitary Development, and Testosterone Synthesis

In our previous study, HIR patients showed a significant reduction of the expression level of genes involved in the HPG axis [Hadziselimovic et al., 2016]. Therefore, we focused on the effects of GnRHa on these genes and found consistent expression patterns, i.e., the expression level was decreased in the HIR group and increased following GnRHa treatment. The GnRHa response includes loci involved in the regulation of gene expression (*DLX3*, *DLX6*, *DLX6-AS1/Evf-2*, *EGR2*, *FOXG1*, *ISL2*, *NHLH2*, *OTX2*, *PAX7*, *POU3F2*, *POU6F2*, *RGS7*, *SOX1*, and *SOX30*), chromatin modification (*PRDM9*), and signal transduction (*NOS1*) (Fig. 1A; online suppl. Tables 1, 2). *FOXG1* was suggested to be involved in GnRH neuron development [Duggan et al., 2008; Garaffo el al., 2015] in a similar fashion to *DLX3*, which acts as a transcriptional activator for both *GnRHR* and *αGSU* [Xie et al., 2013]. GnRHa also increases the expression of *ISL2* and *OTX2* (online suppl. Table 1). *ISL2* is a gene implicated in neural stem cell differentiation and in the derivation of lineage-specific markers. *LHX4* is an important paralog of *ISL2*. OTX2, a homeodomain protein shown to be expressed in murine GnRH neurons, is upregulated during GnRH neuronal development, increases GnRH promoter activity in GnRH-neuronal cell lines, and ensures reproductive competence [Diaczok et al., 2011]. Additionally, *NRG1* and *NR4A2*, 2 genes important in neuronal differentiation and transcriptional regulation [Park et al., 2003], displayed elevated expression following GnRHa treatment (online suppl. Table 1).

Interestingly, a number of transcripts important for pituitary development that were not differentially expressed between the HIR and LIR groups were still upregulated following treatment. They include an lncRNA important for heart and body wall development [Grote et al., 2013], DNA/chromatin binding regulators important for development (*DLX1*, *EGR3*, *FGF5*, *LHX4*, *OTX1*, *PAX1*, *PAX2*, *PAX3*, *PAX4*, *PAX6*, *POU1F1*, *POU2AF1*, *POU2F2*, *POU2F3*, *POU4F2*, *PROP1*, *RUNX2*, *SOX2*), and epigenetic factors involved in histone modification (*PRDM12*, *PRDM16*) [Jostes et al., 1990; Walther and Gruss, 1991; Goulding et al., 1993; Dahl et al., 1997; Kioussi et al., 1999] (Fig. 2; online suppl. Table 1). Moreover, GnRHa increased, as expected, the abundance of several genes that regulate testosterone synthesis (*HSD3B1*, *HSD17B2*,

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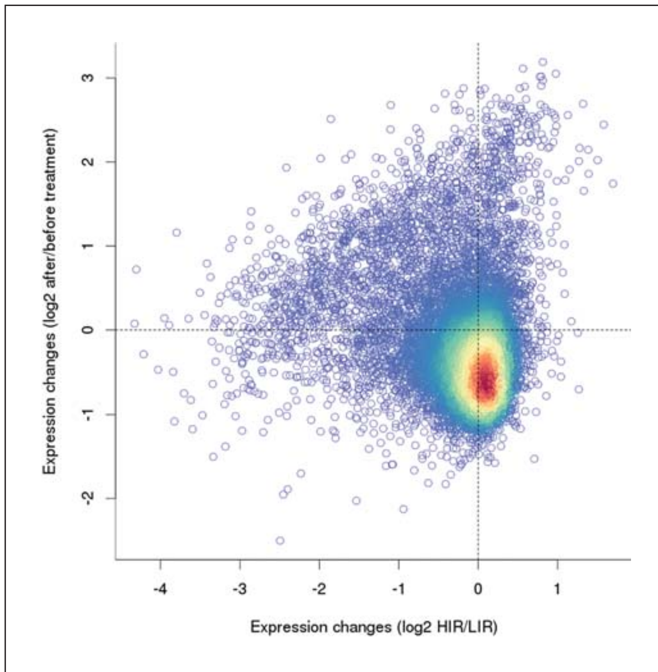

Fig. 2. A plot of the HIR/LIR gene expression changes versus the after/before GnRHa treatment changes. Both HIR/LIR and treatment have specific changes that are unchanged in the other one (genes that are located close to the horizontal or vertical dashed lines), but there are also many genes that are both decreased in HIR and induced by treatment (genes in the upper left of the plot).

CYP19A1, *CYP11B2*, and *CYP11B1*) (online suppl. Table 3). GnRHa stimulates LH secretion and the development of juvenile Leydig cells that, secreting testosterone after LH stimulation, display an increased expression of the steroidogenic enzyme HSD3B [Teerds and Huhtaniemi, 2015]. Thus, the observed increase in *HSD3B1* expression indicates increased testosterone synthesis. Increased *HSD17B2* gene expression yields 17β-HSD type 2 activity that catalyzes the oxidation of androgens and estrogens equally efficiently, while *CYP19A1* encodes the CYP19A1 aromatase, which catalyzes the aromatization of androgens into estrogens. RNA levels for the G-protein coupled receptor class C group 6A (*GPRC6A*) gene involved in the nonclassical testosterone pathway were also upregulated after GnRHa treatment (online suppl. Table 3). Noticeably, GPRC6A is an amino acid, calcium, and osteocalcin sensing G-protein-coupled receptor that has been reported to mediate the nongenomic effects of androgens [Pi et al., 2010; Pi and Quarles, 2013]. Several genes involved in the androgen receptor pathway also showed increased expression, 2 of which encode kallikrein-related serine peptidases (*KLK2* and *KLK3*), with a locus that is extraordinarily sensitive to steroids and other hormones involved in androgen receptor signaling. In addition, following treatment, increased levels of RGS7 (regulator of G-protein signaling 7), *AKR1B15*, and *AKR1D1*, were observed (online suppl. Table 3). As a regulator of G-protein-coupled receptor signaling cascades, *RGS7* is implicated in the control of hormone release and endocrine functions [Nini et al., 2012]. *AKR1B15* is a novel member of the AKR superfamily, and its protein plays a potential role in steroid metabolism, the regulation of mitochondrial function, and aging [Weber et al., 2015]. In the HIR group of cryptorchid testes, this gene was underexpressed in comparison to the LIR group (online suppl. Table 3). The AKRD1D1 enzyme efficiently catalyzes the reduction of progesterone, androstenedione, 17-alpha-hydroxyprogesterone, and testosterone to 5-beta-reduced metabolites. Finally, we also found that expressions of several genes involved in testosterone synthesis were downregulated, e.g., *CYP17A1*, *CYP11A1*, *CYPB5B*, and *HSD17B4/B1/B12/B11* (online suppl. Table 3). GnRHa treatment also stimulated the expression of *FGF23*, which is involved in nonclassical signaling by androgens, with its physiological effects limited to organs that coexpress the FGFR/α-Klotho complexes such as the pituitary gland [Urakawa et al., 2006; Pi and Quarles, 2013].

GnRHa-Dependent Stimulation of HPG Axis Genes

We found that differential gene expression patterns between HIR/LIR patients and GnRHa-treated/untreated patients are partially similar. In addition, GnRHa treatment appeared to stimulate in many cases alternative pathways or entire sets of gene families or their homologues. For example, the HIR group showed reduced RNA levels compared to LIR for bone morphogenetic proteins *BMP6*, *BMP7*, and *BMP8A* (online suppl. Table 1), but their expression is unaltered by GnRHa. However, we observed a 2- to 4-fold increase in the RNA levels of *BMP3*, *BMP5*, and *BMP10* following GnRHa treatment. Similar patterns were found for the family of chemokine ligands (CXCL) and receptors (CXCR).

As opposed to that, GnRHa treatment failed to induce any transcriptional changes in *CXCL9* and *CXCR4* gene expression, both of which were reduced in the HIR compared to the LIR group. Instead, GnRHa treatment increased gene expression for *CXCL3/5/6/8/11/13*, as well as for the chemokine receptors *CXCR1/2/5/6*. In some cases, GnRHa treatment could both boost the expression of certain genes that were found to have a reduced expression in HIR patients and increase the expression of their family members, e.g., for the *DLX* family members *DLX1/3/6* and *EGR* family members *EGR2*

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and *EGR3*; furthermore, for *ISL2*, *NR4A2*, *OTX1*, and *OTX2*, the listed POU class family members, PR domain containing genes, for *RUNX1* and *RUNX2*, *SIX2* and *SIX3*, as well as for *LEP*, *PCSK1*, and *TAC3*, and finally for the *SOX* family.

GnRHa Treatment Affects lncRNAs

Known functions of some nuclear lncRNAs are to regulate gene expression and genome stability. We found that, among others, *AIRN*, *DLX6-AS1*, *FENDRR*, *ERICH1-AS1*, *HAGLR*, *HOTAIR*, *OTX2-AS1*, *IGF2BP2-AS1*, *SLC7A11-AS1*, and *TSIX* displayed an elevated expression following GnRHa treatment (online suppl. Table 2). This is an intriguing preliminary finding that indicates a potentially important role for lncRNAs in the overall response to GnRHa treatment during pre-pubertal testis development.

Working under the assumption that divergently transcribed genes can be functionally linked [Grote and Herrmann, 2013; Grote et al., 2013], Grote et al. [2013] suggested that *Fendrr* (Foxf1 adjacent noncoding developmental regulatory RNA) regulates *Foxa1* and other genes via a Polycomb-dependent epigenetic mechanism. *FENDRR* was the first lncRNA demonstrated to be essential for mammalian embryogenesis [Grote et al., 2013; Dey et al., 2014]. Notably, *Fendrr* interacts with WDR5 [Grote et al., 2013], which is well known for its presence in the MLL complexes that mediate H3K4 methylation, a mark thought to counteract H3K27me.

XIST inactivation is an early developmental process in mammalian females that transcriptionally silences one of the pair of X chromosomes, thus providing dosage equivalence between males and females. This process is regulated by several factors, including a region of the X chromosome called the X inactivation center (XIC) [Loos et al., 2016]. The *TSIX* gene expresses a noncoding antisense transcript (*XIST* Antisense RNA/ *TSIX* transcript) across the 3′ end of the *XIST* (X Inactive Specific Transcript) locus. This antisense RNA was shown to be coexpressed with *Xist* derived solely from the inactive X chromosome in reporter mouse embryonic stem cells [Loos et al., 2016].

AIRN (antisense of IGF2R non-protein coding RNA) and *HOTAIR* (HOX transcript antisense RNA) are both involved in histone modifications [Nagano et al., 2008; Gupta et al., 2010]. The *Airn* macro ncRNA is the master regulator of imprinted expression in the *Igf2* receptor (*Igfr*) imprinted gene cluster, where it silences 3 flanking genes in cis [Stricker et al., 2008]. *Airn* expression not only induces silencing of flanking mRNA genes but also protects the paternal copy of *ICE* from de novo methylation. *HOTAIR*, transcribed from within the *HOXC* gene cluster, was shown to play a repressive role at the *HOXD* locus by interacting with PRC2 [Zhang et al., 2014]. In addition, *HOTAIR* also interacts with the histone H3K4me1/2 demethylase LSD1 (KDM1), which removes a histone mark indicative of active chromatin, thus reinforcing the establishment of a repressive chromatin environment on its target loci [Rinn et al., 2007]. Noticeably, HIST3H3 expression was significantly reduced in the HIR group (–3.5 log, FDR 0.001).

GnRHa treatment also increased the expression of *DLX6-AS1* and *OTX2-AS1*. *Dlx6-as1* (*Dlx6* antisense 1, also known as *Evf1*), transcribed from the genomic region between *Dlx5* and *Dlx6*, inhibits the remodeling activity of SWI/SNF (Switching/Sucrose Non-Fermenting), a nucleosome remodeling complex, in order to interfere with the upregulation of *Dlx5*/*Dlx6* [Bond et al., 2009]. OTX2-AS1 is a natural antisense transcript (NAT) RNA that plays an important role in eye development [Alfano et al., 2005]. *OTX2-AS1* displays sequence complementarity to the exon sequences in its corresponding sense gene, *OTX2*, both in mouse and human. *OTX2-AS1* is predominantly expressed in the retina, with a lower level of expression in the brain. NATs are biologically important sources of endo-siRNAs and are essential components of a regulatory network to control the mutagenic burden that arises at the nucleic acid level without direct consequences for protein expression [Werner et al., 2009].

The GnRHa Response Protein Network

We next interpreted our transcript signatures in the context of physical protein-protein interactions and functional interactions by integrating our data with information available in the literature (STRING interaction network; http://string-db.org). Among the differentially expressed genes identified in our study, 146 encode proteins that correspond to the hypothalamic-pituitary-testicular axis and neuronal development. Analyses of the protein-protein interaction network containing these proteins resulted in high confidence interactions scores (≥0.7) for PROP1-POU1F1-PITX1-LHX4 and ISL1, and DLX1-POU4F2-ISL1-OTX2-PAX6 with FOXG1 (Fig. 3). Furthermore, additional strong high-confidence interactions were predicted for the chemokine cluster, with NMU linked to both PROK2 and GPRC6A, and NOS1 connected to EGR2, GATA2, and GATA3. Moreover, we observed high confidence connections between *EGR2* and *GATA3*, and *WNT3*, *EBF1*, *OTX1*, *OTX2*, and *POU3F*.

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Fig. 3. Protein interaction network of genes involved in the HPG axis and differentially expressed in HIR/LIR and after GnRHa treatment. Protein-coding genes upregulated after GnRHa treatment are framed in red. Protein interaction network is predicted using STRING [Szklarczyk et al., 2015] with a confidence cut-off of 0.7. Line-weight represents strength of data support between predicted interactions.

These direct and indirect interactions between proteins encoded by differentially expressed genes are consistent with a coordinated reprogramming of gene expression induced by GnRHa treatment, and they provide an explanation for the observed changes in a large number of genes in different gene families. We note that the nitric oxide synthase-1 gene plays a modulatory role in the biology of a variety of neuroendocrine tissues, with an important role in the regulation of testosterone release [Wang et al., 2002].

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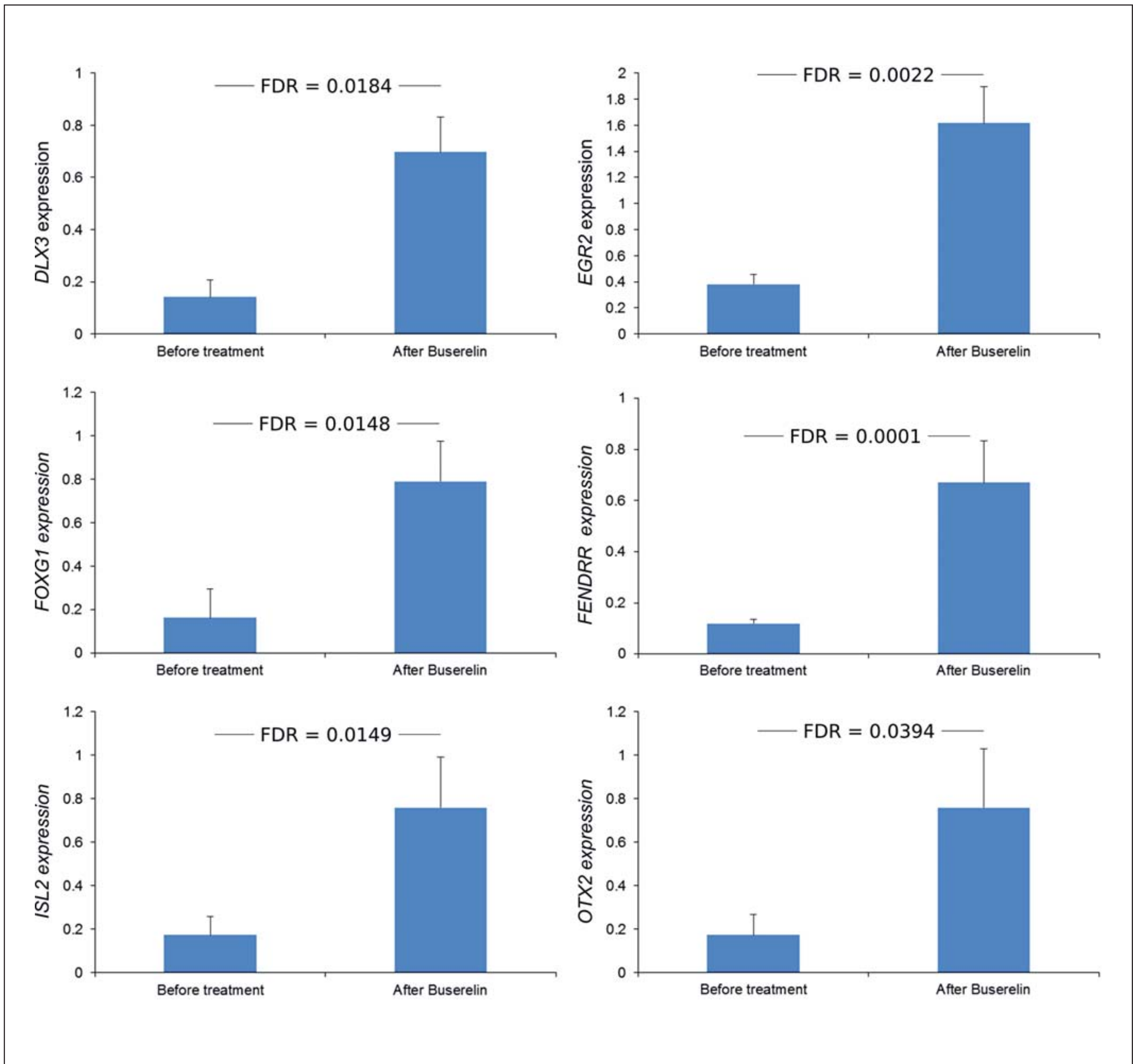

Fig. 4. Expression levels of *DLX3*, *EGR2*, *FOXG1*, *FENDRR*, *ISL2*, and *OTX2* analyzed by RNA sequencing in cryptorchid testes lacking Ad spermatogonia before and after GnRHa/Buserelin treatment. Gene expression is shown as median RPKM values; mean absolute deviations and FDR values are presented.

Discussion

Transcriptome Analysis of Complex Human Tissues

RNA profiling revealed that GnRHa treatment induces a transcriptional response involving genes which control the HPG axis, reproduction, and testosterone synthesis via both the classical and alternate pathways. Furthermore, our analysis showed that several lncRNAs involved in epigenetic programing are responsive to GnRHa. In contrast, surgery alone had no effect on gene expression, underscoring the importance of GnRHa treatment in rescuing fertility.

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Expression studies of human testis are inherently complicated, because a male gonad is a complex mixture of, among others, germ cells at different stages of meiotic development and nurse cells that provide hormonal and nutritional cues critical for the establishment of normal spermatogenesis. These difficulties notwithstanding, we and others have provided ample evidence that testicular expression profiles are informative [Hadziselimovic et al., 2009, 2016; Chalmel et al., 2012]. A critical issue, especially when working with human samples, is the number of cases that are included in a given analysis. First, the number of replicates affects the statistical confidence level, and second, human tissue samples show intrinsic variability that needs to be controlled for. In this exploratory RNA profiling study, we have included 7 patients taken sequentially from a large ongoing study that is based on randomized patient samples. That is to say, their inclusion in the cohorts to be treated or to remain untreated was completely unbiased by any parameter other than undescended testes which were surgically corrected. Four samples from GnRHa-treated patient were compared to 3 samples from untreated controls. This sample size, while small, is sufficient for an initial transcriptome study as we present it here.

The Differential RNA Profiles of HIR/LIR versus GnRHa-Treated/Untreated Testicular Biopsies Partially Overlap

We found that genes unaffected in HIR patients but important for pituitary development were also upregulated after treatment (online suppl. Table 1). One factor that plays a crucial role in neural precursor cell proliferation and maintenance is *SOX2* (SRY-Box 2) [Graham et al., 2003]. In rat, *PROP1* (Prophet of PIT1) is consistently expressed in SOX2-expressing stem/progenitor cells in the pituitary [Yoshida et al., 2009]. Furthermore, it was shown that *SOX2* is able to modulate *PROP1* expression and that various transcription factors might participate in the regulation of *PROP1* in a *SOX2*-dependent or independent manner. *PROP1* is a pituitary-specific transcription factor that plays an important role in pituitary organogenesis and in the differentiation of hormone-producing cells [Nishimura et al., 2016]. In this context, it is intriguing that both *SOX2* and *PROP1* gene expression was elevated after GnRHa treatment (online suppl. Table 1). *SOX2* is also linked to the PR domain containing proteins *PRDM14* and *PRDM16*. PRDM family proteins interact with the Notch-Hes pathway during neurogenesis and may control both nervous system patterning and the modulation of neuronal progenitor cell proliferation and differentiation [Kinameri et al., 2008]. Given the 4 upregulated *PRDM* genes (*PRDM7*/*9*/*12*/*16*), a provocative potential link between Prdm14 function and Prdm16 is that Prdm14 mediates the acquisition of germ cell pluripotency, in part by upregulating Sox2 [Yamaji et al., 2008]. *PRDM* factors act either as direct histone methyltransferases or recruit a suite of histone-modifying enzymes to target promoters and modify histone function [Hohenauer and Moore, 2012]. The absence of GnRHa stimulation of *EGR1* and *EGR4* remains an enigma. These (potentially) epigenetically downregulated genes appear not to be responsive to GnRHa treatment at the level of mRNA. Nevertheless, the observed LH increase after 6 months of therapy [Hadziselimovic et al., 1984] would suggest that LH secretion is the result of alternate pathway stimulation via *EGR2* and *EGR3*, together with *PROP1* (Fig. 4). Furthermore, the *TAC1* gene, implicated in the control of the GnRH pulse generator and thus LH secretion, plays a critical role in the neuroendocrine regulation of reproduction, mediating its biological effects via 3 G-protein-coupled receptors [Lasaga and Debeljuk, 2011; Kalil et al., 2016]. The observed increases in *TAC1* and *TAC3* gene expression following GnRHa treatment indicates that both their protein products, together with the *EGRs*, *PROK2*, *PROP1*, *LEP*, and PITX1, may participate in orchestrating LH secretion (online suppl. Table 1).

Are lncRNAs Critical for the Curative Effect Observed after GnRHa Treatment?

Our preliminary data indicate that GnRHa treatment affects the levels of numerous lncRNAs including some that are likely to be biologically relevant for human reproduction. For example, the antisense lncRNA OTX2-AS1, which reacts positively to GnRHa stimulation, is interesting. While no role has been found for it, deletion of its sense gene *OTX1* was found in 6 subjects with genitourinary defects. Three of these individuals were diagnosed with cryptorchidism [Jorgez et al., 2014]. Moreover, *Otx2* heterozygous male mice display compromised fertility (reduced LH levels and testicular weight) due to a defect in the development, number, and migration of GnRH neurons [Larder et al., 2013]. Otx1 and Otx2 have functional similarity and interchangeable roles [Acampotra et al., 1999], and Otx2 could compensate for Otx1 deficiency in levels that vary among subjects. Any potential roles for lncRNAs in GnRHa treatment effect remain speculative for the moment, but our results clearly indicate that they are potentially important targets for further analyses.

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Conclusion and Outlook

GnRHa treatment stimulates the expression of genes involved in pituitary development and differentiation as well as neuronal development and loci involved in the canonical and alternative testosterone synthesis pathways. Critically, we now show for the first time that lncRNAs involved in the epigenetic control of gene expression are also upregulated after treatment. This finding is consistent with the idea that hypogonadotropic hypogonadism in the HIR group of cryptorchid boys might be epigenetically programmed. Our results provide initial insight into the molecular events that underlie GnRHa-mediated germline development and postnatal male gonad maturation in HIR patients. These data, while preliminary, are consistent with previous histological and clinical observations of GnRHa treatment. More work is needed to unravel the molecular mechanisms behind cryptorchidism-related adult infertility.

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Statement of Ethics

In accordance with the Declaration of Helsinki, the Institutional Review Board and the Independent Ethics Committee of Vilnius University approved all aspects of this study. Approval was also provided for research involving the use of material (data records or biopsy specimens) that had been collected for non-research purposes (Vilnius Regional Biomedical Research Ethics Committee, No. 158200-580-PPI-17).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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