Testicular expression of long non–coding RNAs is affected by curative GnRHa treatment of cryptorchidism

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Abstract

Background: Cryptorchidism is a frequent endocrinopathy in boys that has been associated with an increased risk of developing testicular cancer and infertility. The condition is curable by combined surgery and hormonal treatment during early pre-pubertal stages using gonadotropin releasing hormone agonist (GnRHa). However, whether the treatment also alters the expression of testicular long non-coding RNAs (lncRNAs) is unknown. To gain insight into the effect of GnRHa on testicular lncRNA levels, we re-analyzed an expression dataset generated from testicular biopsies obtained during orchidopexy for bilateral cryptorchidism.

Results: We identified EGFR-AS1, Linc-ROR, LINC00221, LINC00261, LINC00282, LINC00293, LINC00303, LINC00898, LINC00994, LINC01121, LINC01553, and MTOR-AS1 as potentially relevant for the stimulation of cell proliferation mediated by GnRHa based on their direct or indirect association with rapidly dividing cells in normal and pathological tissues. Surgery alone failed to alter the expression of these transcripts.

Conclusion: Given that lncRNAs can cooperate with chromatin-modifying enzymes to promote epigenetic regulation of genes, GnRHa treatment may act as a surrogate for mini-puberty by triggering the differentiation of Ad spermatogonia via lncRNA-mediated epigenetic effects. Our work provides additional molecular evidence that infertility and azoospermia in cryptorchidism, resulting from defective mini-puberty cannot be cured with successful orchidopexy alone.

Keywords: Spermatogenesis, Cryptorchidism, Male infertility, GnRHa, IncRNAs, Antisense IncRNAs, lincRNAs, Mitosis

Résumé

Contexte: La cryptorchidie est une endocrinopathie fréquente chez les garçons. Elle est associée à un risque élevé de cancer des testicules et d’infertilité. La cryptorchidie peut être soignée par une thérapie incluant une intervention chirurgicale et un traitement hormonal par l’agoniste de l’hormone GnRH. Alors que l’effet de la thérapie sur l’expression des ARNm a été analysé, ses conséquences pour la transcription des longs ARNs non codants (ARNlnc) testiculaires restent inconnues. Afin de mieux comprendre les effets du GnRHa sur les concentrations cellulaires des ARNlnc dans le testicule, nous avons analysé des données d’expression d’ARN par séquençage (ARN-Seq) générées en utilisant des biopsies testiculaires obtenues dans le cadre d’une orchidopexie pour cryptorchidie bilatérale.

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Introduction

Long non-coding RNAs (lncRNAs) have emerged as key regulators of gene expression in embryonic stem-cell (ESC) self-renewal and differentiation. In ESCs, lncRNAs are regulated at the genetic level by transcription factor binding to lncRNA gene promoters. A major function of lncRNAs is the regulation of specific gene expression at multiple steps, including the recruitment and expression of basal transcription machinery, post-transcriptional modifications, and epigenetics [1]. LncRNAs have also been proposed to play a targeting role by binding to certain methyltransferases and demethylases, and directing them to specific genomic locations. Depending on the biological context, certain methylation events are stably maintained (e.g., methylation involved in inheritance through mitosis of a silenced heterochromatin state), whereas others have to be amenable to change (e.g., when cells differentiate or respond to environmental cues) [2–5]. The so-called natural antisense transcripts (NATs) have been shown to regulate gene expression by affecting transcription and mRNA stability [2–5]. Almost 80% of the mammalian genome is transcribed, and many genomic loci produce RNAs from both sense and antisense DNA strands [6–8], though the functional importance of most of these transcripts is only poorly characterized.

We have previously demonstrated that the presence of type A dark (Ad) spermatogonia in the testis is a marker of low infertility risk (LIR), whereas low or absent levels (below a critical threshold) indicate high infertility risk (HIR) [9, 10]. Treatment with a gonadotropin-releasing hormone agonist (GnRHa, buserelin) enables the Ad spermatogonia population to recover, significantly improving fertility in HIR patients [11]. GnRHa induces a broad transcriptional response, including genes encoding proteins involved in pituitary development, the hypothalamic-pituitary-gonadal axis, and testosterone synthesis [12]. Earlier work focused on protein-coding mRNAs; consequently, nothing is known about the expression of lncRNAs in the treatment of cryptorchidism.

We identified several hundred GnRHa-responsive lncRNAs, which were grouped into long intergenic non-coding RNAs (lincRNAs) and antisense (AS) lncRNAs. We selected candidates on the basis of their expression profiles and then prioritized them for roles in cell growth, differentiation, and disease based on a literature search in PubMed (www.ncbi.nlm.nih.gov/pubmed/). We also included in this search protein-coding genes located upstream or downstream lincRNAs and sense genes overlapping AS-lncRNAs. In addition, we explored the RNA-RNA interaction data available in the RISE database (http://rise.life.tsinghua.edu.cn). Finally, we interpreted lncRNA expression signals in the context of protein/RNA profiling data published by the Human Protein Atlas (www.proteinatlas.org) and RNA-sequencing data from HIR/LIR patients [12]. We propose that certain hormone-responsive lncRNAs may play a role in establishing adult spermatogenesis during pre-pubertal stages of development by controlling testicular cell proliferation.

Materials and methods

Study population and biopsy sample collection

The samples used in this study have been described elsewhere [12–14]. A cryptorchid testis is defined as a testis localized outside of the scrotum and incapable of being brought into a stable scrotal position. Sixteen boys with isolated bilateral cryptorchidism who underwent orchiopexy were prospectively included in this study (Fig. 1). The patients had a median age of 18.5 months (range 8–59 months). During the first orchiopexy, biopsies of the ipsilateral testicle were obtained from all patients. Based on histological evaluation, biopsies were categorized into two groups, Ad- (or HIR) and Ad+ (or LIR). The Ad- group included biopsies with no Ad, and the Ad+ group included testes with Ad spermatogonia (Fig. 1). Cryptorchid boys in the Ad-
group had 8-times lower plasma LH levels (0.11 IU/L) than the Ad+ group (0.89 IU/L, \( p < 0.009 \)), indicating hypogonadotropic hypogonadism [13]. Five boys (Ad-group) were randomly included in each arm. One HIR patients was excluded (Fig. 1.) In the GnRHa-treated group, the median total germ cell count per tubule (S/T) increased from 0.11 to 0.42 (\( p = 0.03 \), paired-samples Wilcoxon test, one-tailed). In the surgery only group, the median S/T did not change and none of the testes had Ad spermatogonia. In contrast, in the GnRHa treated group, all testes completed the transition from gonocytes to Ad spermatogonia (\( p = 0.008 \); Fisher test, 2-tailed) [14].

RNA-sequencing data analysis

The workflow from RNA isolation to purification, library preparation, sequencing, data analysis, and expression level analysis has been described in detail elsewhere [12]. Determination of differentially expressed genes, statistical analyses, and model design were carried out as described previously [12]. Genomic coordinates for known IncRNAs were obtained from the Bioconductor package TxDb.Hsapiens.UCSC.hg19.lincRNAsTranscripts (version 3.2.2). 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 with a
false discovery rate (FDR) of less than 0.05. Raw data files are available at the Database of Genotypes and Phenotypes (dbGaP) under accession number phs001275.v1.p1. Expression signals are given in standard RKPM units. They are calculated as follows: the number of reads mapped to a gene sequence is divided by the length of the gene sequence over 1000 multiplied by the total number of mapped reads per sample over 1’000’000.

LncRNA data interpretation
We analyzed lncRNA and AS-lncRNA expression in cryptorchid patients with HIR before and after GnRH treatment to identify all RNAs annotated as antisense (AS) transcripts, as well as all RNAs annotated as lncRNAs with logFC > 1.0. In addition, we compared lincRNA and AS- lncRNA expression between the HIR and LIR groups of cryptorchid patients and analyzed those with lower expression in the HIR group. LincRNAs and AS- lncRNAs were prioritized based on RNA-RNA interactions, revealing the lncRNAs, AS- lncRNAs, or mRNAs encoding proteins involved in spermatogenesis or fertility, and important IncRNAs or mRNAs encoding proteins involved in cell division/growth, signaling pathways, and cancer. Furthermore, we included five lincRNA directly related to spermatogenesis that had a log FC > 1.0. After the AS-lncRNA candidates were identified, they were prioritized based on a PubMed literature search of themselves and their overlapping sense mRNA/protein, revealing roles in spermatogenesis, fertility, cell division/growth, signaling pathways, and cancer (www.ncbi.nlm.nih.gov/pubmed). The RNA annotation was verified using Ensembl (www.ensembl.org; release 97). The lincRNA/mRNA expression was interpreted using GermOnline (www.germonline.org; version 4.0), Human Protein Atlas (www.proteินatlas.org; version 18), and Genevestigator (www.genevestigator.com; version 7.3.1). Experimentally validated RNA-RNA interaction data were retrieved from RISE (http://rise.life.tsinghua.edu.cn; version 1.0).

Results
Global effects on testicular lncRNA levels in response to GnRH treatment
First, we identified significantly differentially expressed lncRNAs in duplicate testicular biopsies from LIR and HIR patients who underwent surgical correction of undescended testis (Fig. 2, lanes 1–4).

Next, we compared samples obtained from HIR patients at the time of initial surgery (Fig. 2, lanes 5 and 6) and after six months of treatment with GnRHα (Fig. 2, lanes 7 and 8). The genes were ordered using an unsupervised clustering method (hierarchical clustering with complete linkage using Euclidean distances) and are shown in a false-color heatmap relative to the mean expression of each gene over all samples in Fig. 2. The results indicate that a large number of lncRNAs accumulate at low levels in the testes of boys with HIR compared to LIR, and that a substantial fraction of these transcripts is up-regulated by GnRHα treatment. In contrast, surgery alone had no significant impact on lncRNA expression. We explored the dataset using Volcano plots that display statistical significance (false discovery rate, FDR) against fold-change of expression signals allowing the selection of genes for which large and significant differences in expression levels were observed (Fig. 3).

We found that 627 and 38 lncRNAs were expressed at lower and higher levels, respectively, in HIR versus LIR samples (Fig. 3a). We concluded that the vast majority of differentially expressed lncRNAs are detected at lower levels in HIR testes. Comparing HIR testes before and after GnRHα treatment, we found that 3074 lncRNAs were increased, whereas 53 were decreased (Fig. 3b). Thus, hormonal treatment induces a considerable number of lncRNAs. In the following section the novel lncRNAs were organized based on their known functions or roles that were attributed to their potential target genes.

Certain testicular lncRNAs upregulated by GnRHα treatment are involved in stem cell renewal, signaling, and cell differentiation.
We also sought to gain insight into the potential roles that hormone-responsive RNAs might play by interpreting their genomic location, association with protein coding genes in sense/antisense pairs, and RNA-RNA interactions. We selected 11 of 77 lincRNAs and four of 46 AS-lncRNAs with >2.0-fold change after GnRHα treatment because their expression patterns lead us to hypothesize that they are important for the development of Ad spermatogonia (Table 1). In this section we focus on novel potential regulatory lincRNAs and we provide context information about their putative protein-coding target genes. This includes previously published expression data obtained with samples from HIR and LIR patients (fold change and FDR values) [12] and functional information relevant for germ cells growth and differentiation from the literature.

LINC01016 is a so-called hub RNA that binds many mRNAs (encoding epigenetic regulators and transcription factors) and lncRNAs (including XIST). This feature distinguishes a hub RNA from most other transcripts that interact with few, one or no other RNAs. LINC01016 is expressed in the same direction as MLN (Motilin) and is a transcriptional target of the estrogen receptor [15] (Table 1).

LINC01121 is expressed upstream of SIX2 and may influence its proximal promoter regions. SIX2 interacts with TCF7L2 and OSR1 in a canonical WNT signaling-independent pathway, preventing the transcription of
Fig. 2 (See legend on next page.)
differentiation genes in cap mesenchyme, such as WNT4 [16–18] (Table 1).  

LINC00261 is expressed in the 3’regions of PAX1 and FOXA2, which encode transcription factors. It is also a hub IncRNA that binds mRNAs and lncRNAs, including HOTAIR, and is an epigenetically regulated tumor suppressor that is essential for activation of the DNA damage response [19]. FOXA2 is involved in androgen receptor regulation [19–21] and upregulated after GnRHa treatment (log2FC = 1.69; FDR = 0.0004). Furthermore, LINC00261 is a tumor suppressor that blocks cellular proliferation by activating the DNA damage response [22].  

LINC00303 is expressed upstream of SOX13, a developmental factor expressed in mouse Leydig cells and germ cells [23]. Therefore, this lncRNA could be involved in SOX13 regulation. LINC00293 is expressed upstream of SPIDR, which is involved in double-stranded break repair and genome integrity and binds two TTTY type testis-specific lncRNAs. Several lncRNAs involved in DNA damage repair were increased after GnRHa treatment, including LINC00994 (expressed upstream of PSMD6), LINC00898 (binds mRNA encoding USP1), and testis-specific LINC01553 (interacts with mRNA encoding TIMELESS). TIMELESS plays an important role in the control of DNA replication, the maintenance of genome stability throughout normal DNA replication, and regulation of the circadian clock [24]. (Table 1).  

EGFR-AS1, which is involved in determining period length and in the DNA damage-dependent phase advancing the circadian clock [25], interacts with NEU3 mRNA. NEU3 activity enhances EGFR activation without affecting EGFR expression. This may indicate a regulatory mechanism involving a feedback loop. EGFR-AS1 is weakly expressed in adult testis and highly expressed in liver and liver cancer. Intense EGFR immunostaining was found in men with high plasma FSH levels and in all patients who received exogenous FSH, supporting a possible gonadotropin role in the modulation of EGFR expression [25]. GnRHa treatment increased the plasma FSH level and EGFR-AS1, but decreased EGFR expression (log2FC = −0.58; FDR = 0.01). Epidermal growth factor receptor signaling is associated with the pathogenesis of

Fig. 2 RNA-sequencing data for IncRNAs. A false color heatmap (red is high, blue is low) shows data from pairs of testes, analyzed. Horizontal bars at the top indicate patient categories and age. Four sample from two HIR patients (p32 and p33; biopsy number) having had surgery “only” treatment; line 1 and 2 during first surgery obtained from ipsilateral testis (I), line 3 and 4 show results from contralateral testis six months after first orchidopexy (II) Buserelin treated patients p5 and p6; line 5 an 6 before treatment and line 7 and 8 contralateral testis after hormonal treatment. Color scales for expression (red/blue) and age (green) are shown.

Fig. 3 Volcano plots of IncRNA expression ratios: (a) Between the high (HIR) and low infertility risk (LIR) groups or (b) in the HIR group before and after GnRHa treatment. Genes with no significant difference in expression between the two groups compared in each panel are in black. Differentially expressed genes are shown in red. The most upregulated genes on the right, the most downregulated genes on the left, and the most significant genes at the top.
cutaneous squamous cell carcinoma. LINCO0520-targeted EGFR inhibition might result in inactivation of the PI3K/Akt pathway, thereby inhibiting cancer development [26].

HOTTIP mediates the regulation of CXCL genes, which are implicated in Ad spermatogonia differentiation [12]. HOTTIP is antisense to HOXA13 and modulates cancer stem cell properties in human pancreatic cancer by regulating HOXA9 [27, 28]. OTX2-AS1 is a NAT RNA that plays an important role in eye development and exhibits sequence complementarity to the exon sequences in its corresponding sense gene, OTX2, in both mice and humans [12]. OTX2 is downregulated in HIR (log2FC = −1.73; FDR = 0.02) and upregulated after GnRH treatment (log2FC = 1.24; FDR = 0.03) [12]. Though no role has been found for OTX2-AS1, deletion of its sense gene OTX1 was found in six patients with genitourinary defects. Three of these individuals were diagnosed with cryptorchidism [29]. MTOR, the key regulator of spermatogenesis [30], is downregulated in boys with HIR (log2FC = −0.42; FDR = 0.03) and remains downregulated after GnRH treatment (log2FC = −0.53; FDR = 0.02). Its antisense gene, MTOR-AS1, was up-regulated 4.9-log2 by GnRH treatment (Table 1). Thus far, nothing is known about the function of MTOR-AS1.

LINC-ROR is induced 6.5-fold by GnRHa. (Table 1) This IncRNA controls stem cell renewal and acts as an miRNA sponge via gene silencing, which indicates that the transcript itself has a biological role [31–33]. In addition, we found that BOD1L2, a testis-specific gene, is located downstream of LINC-ROR and may be transcriptionally regulated by the IncRNA. BOD1L2 plays a role in chromosome biorientation through the detection or correction of syntelic attachments in mitotic spindles [34, 35]. Buserelin treatment increases BOD1L2 gene expression (log2FC = 1.72; FDR = 0.003), indicating a possible role for it in Ad spermatogonia differentiation.

LncRNAs downregulated in HIR testes and stimulated after GnRHa treatment are associated with cancer and the transition of ad spermatogonia

We previously reported different lncRNA expression in patients with HIR compared to LIR; some of these RNAs participate in epigenetic processes, including AIRN, ERICH-AS1, FENDRR, HAGLR, and XIST [12]. Here, we focus on seven lncRNAs with decreased gene expression in HIR, indicating abrogated mini-puberty, and increased expression after GnRHa treatment (Tables 2 and 3).

Table 1 Testicular lncRNAs and AS-lncRNAs that increase after GnRHa treatment and are involved in stem cell renewal and differentiation

| Gene ID     | RPKM before GnRHa Median MAD | RPKM after GnRHa Median MAD | log2FC GnRHa | p-value | FDR |
|-------------|-------------------------------|-------------------------------|--------------|---------|-----|
| LINC-ROR    | 0.044 / 0.06                 | 0.40 / 0.24                  | 2.68         | 0.0004  | 0.002|
| LINCO0261   | 0.11 / 0.04                  | 1.11 / 0.68                  | 2.60         | 3.588E-08 | 4.35E-06|
| LINCO0293   | 0.05 / 0.04                  | 0.66 / 0.49                  | 2.80         | 0.0001  | 0.001|
| LINCO0303   | 0.22 / 0.07                  | 1.25 / 0.45                  | 2.57         | 0.0001  | 0.0008|
| LINCO0520   | 0.15 / 0.07                  | 0.79 / 0.64                  | 2.76         | 0.0002  | 0.001|
| LINCO0898   | 0.04 / 0.03                  | 0.35 / 0.25                  | 2.72         | 0.0002  | 0.001|
| LINCO0974   | 0.07 / 0.04                  | 0.48 / 0.46                  | 3.9          | 0.0008  | 0.003|
| LINCO0994   | 0.20 / 0.10                  | 1.41 / 0.81                  | 2.73         | 5.773E-06 | 0.0001|
| LINCO1016   | 0.15 / 0.03                  | 1.32 / 0.70                  | 3.36         | 2.078E-09 | 6.43E-07|
| LINCO1121   | 0.25 / 0.04                  | 1.84 / 0.45                  | 2.89         | 2.049E-09 | 6.43E-07|
| LINCO1553   | 0.09 / 0.06                  | 1.26 / 0.67                  | 3.60         | 0.0002  | 0.001|
| EGRF-AS1    | 0.03 / 0.05                  | 0.58 / 0.30                  | 2.99         | 3.850E-07 | 2.28E-05|
| HOTTIP      | 0.04 / 0.03                  | 0.47 / 0.37                  | 2.22         | 0.0010  | 0.004|
| MTOR-AS1    | 0.21 / 0.19                  | 1.90 / 1.33                  | 4.96         | 6.67E-06 | 0.0001|
| OTX2-AS1    | 0.09 / 0.007                 | 0.91 / 0.45                  | 2.37         | 2.95E-06 | 8.83E-05|

The log-fold changes (FC), p-value, false discovery rate (FDR), median expression values in reads per kilobase per million (RPKM) (Median), and the median absolute deviation (MAD) for LINC samples before and after GnRHa treatment are given.
modulating the miR-489-3p/TPT1
tion of SRY repressor in fetal testes, contributing to the precise regula-
SOX4 together with (log2FC = 0.7; FDR = 0.008). It has been suggested that, to-
ing. SOX11 is important for embryonic neurogenesis and tissue model-
absolute deviation (MAD) for LINC samples before and after GnRHa treatment are shown

The log-fold changes (FC), p-value, false discovery rate (FDR), median expression values in reads per kilobase per million (RPKM) (Median), and the median absolute deviation (MAD) for LINC samples are presented.

LINC01249 is expressed upstream of SOX11, which is important for embryonic neurogenesis and tissue modeling. SOX11 is upregulated after GnRHa treatment (log2FC = 0.7; FDR = 0.008). It has been suggested that, together with SOX4, SOX11 may function as a transcriptional repressor in fetal testes, contributing to the precise regulation of SRY and SOX9 [23].

LINC01446 promotes glioblastoma progression by modulating the miR-489-3p/TPT1 pathway [38].

The testis expression of LINC00701 is developmental stage-specific and associated with SLC25A37, encoding a solute carrier localized in the inner mitochondrial membrane. The protein functions as an essential iron importer for the synthesis of mitochondrial heme and iron-sulfur clusters [39].

HOX antisense intergenic RNA (HOTAIR) is an lncRNA that coordinates with chromatin-modifying enzymes, regulates gene silencing, and is transcriptionally induced by estradiol (E2) [12, 40, 41].

Distal-less homeobox6 antisense (DLX6-AS1) was downregulated in HIR and responded positively to GnRHa treatment. This supports the observation in mice that DLX6 participates in the control of steroidogenesis [42]. DLX5 and DLX6 showed low or no expression in HIR samples.

TINCR, an lncRNA required for the induction of key differentiation genes, is downregulated in HIR testes (log2FC = −1.07; FDR = 0.002). Seven epigenetic modifiers found to bind TINCR were upregulated in HIR and downregulated after GnRHa treatment (Table 4).

**Discussion**

In this study, we aimed to gain molecular insight into the effect on testicular lncRNA expression levels of a curative treatment for cryptorchidism and related infertility that combines surgery and nasal administration of GnRHa [11, 12, 14]. We found hundreds of lncRNAs that respond to treatment, including a subset that is present at lower levels in testicular samples from boys with HIR. A detailed interpretation of the expression data revealed candidate lncRNAs that may play important regulatory roles in establishing adult spermatogenesis during early postnatal development in humans. Our 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. The results point to molecular mechanisms that underlie the ability of GnRHa to rescue fertility.

**Study design for human testicular RNA profiling experiments**

When working with human samples, a critical issue is the number of cases included in a given analysis. The number of replicates affects the statistical confidence level, and human tissue samples exhibit intrinsic variability that needs to be controlled. In this exploratory lncRNA profiling study, we included first seven patients chosen sequentially from a study based on randomized patient samples [12, 14]. 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. This sample size, while small, is enough for an initial transcriptome study as presented here.

**Table 2** Testicular lncRNAs downregulated in HIR testes compared to LIR

| lncRNA | LIR Median/MAD | HIR Median/MAD | log2FC | p-value | FDR |
|--------|----------------|----------------|--------|---------|-----|
| LINC00922 | 0.47 0.17 | 0.10 0.07 | -1.47 | 0.002 | 0.01 |
| LINC00221 | 0.64 0.27 | 0.27 0.07 | -1.20 | 0.0007 | 0.008 |
| LINC01249 | 0.43 0.28 | 0.15 0.08 | -1.42 | 0.001 | 0.01 |
| LINC00701 | 0.29 0.03 | 0.11 0.06 | -0.84 | 0.002 | 0.02 |
| HOTAIR | 0.49 0.23 | 0.13 0.09 | -1.74 | 0.0001 | 0.002 |
| DLX6-AS1 | 0.42 0.09 | 0.20 0.06 | -0.92 | 0.006 | 0.03 |
| LINC01446 | 0.57 0.09 | 0.31 0.08 | -1.21 | 0.0002 | 0.003 |

The log-fold changes (FC), p-value, false discovery rate (FDR), median expression values in reads per kilobase per million (RPKM) (Median), and the median absolute deviation (MAD) for LINC samples before and after GnRHa treatment are shown.

**Table 3** LncNRAs downregulated in HIR testes and stimulated after GnRHa treatment

| lincRNA (RPKM) | before GnRHa | after GnRHa | log2FC | p-value | FDR |
|----------------|--------------|-------------|--------|---------|-----|
| LINC00922 | 0.10 0.07 | 0.85 0.40 | 1.41 | 0.02 | 0.04 |
| LINC00221 | 0.27 0.07 | 1.12 0.42 | 1.23 | 0.01 | 0.03 |
| LINC01249 | 0.15 0.08 | 0.92 0.56 | 1.38 | 0.003 | 0.01 |
| LINC00701 | 0.11 0.06 | 0.72 0.48 | 1.28 | 0.0009 | 0.003 |
| HOTAIR | 0.13 0.09 | 1.04 0.87 | 1.14 | 0.01 | 0.03 |
| DLX6-AS1 | 0.20 0.06 | 1.13 0.81 | 2.02 | 1.55E-05 | 0.0002 |
| LINC01446 | 0.31 0.08 | 1.49 0.82 | 1.14 | 0.0007 | 0.003 |
Curative hormone treatment affects signaling pathways

During GnRHa treatment, increased LH and testosterone secretion induced the transition of gonocytes and undifferentiated spermatogonia into Ad spermatogonia. In this context, it is interesting that the expression of LINC-ROR, a key regulator of pluripotent stem cell reprogramming, increased after hormone treatment. LINC-ROR influences cell differentiation, in part, by acting as a sponge for miR-138 and miR-145 and by LINC-ROR influences cell differentiation, in part, by reprogramming, increased after hormone treatment.

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Hormone treatment influences epigenetic factors

LINC00261 stimulates the expression of HOXAI and HOXTIP genes to stabilize androgen receptor (AR) together with FOXA1. Specifically, the DNA-binding domain (DBD)/hinge region of AR directly interacts with the fork head domain of FOXA1, thereby acting as an AR-collaborating factor.

HOTAIR’s promoter contains multiple functional estrogen response elements (EREs). HOTAIR mediates the recruitment of H3K27 methyltransferase and H3K4 demethylase, which leads to efficient repression of certain loci. The levels of histone H3 lysine-4 trimethylation, histone acetylation, and RNA polymerase II recruitment are increased at the HOTAIR promoter in the presence of E2, and knock-down of ERs downregulated E2-induced HOTAIR expression. Thus, like the transcription of protein-coding genes, E2 induces the transcription of antisense RNAs [27, 49, 50]. HIR patient’s exhibit decreased HOTAIR levels, [12] and GnRHa treatment induced HOTAIR expression, indicating that LH, testosterone and/or converted E2 have a positive effect on HOTAIR and, thus, Ad spermatogonia differentiation [12]. Furthermore, HOTAIR was previously shown to mediate tumorigenesis by recruiting EZH2 [51]. This is of interest, as GnRHa induces expression of HOTAIR, downregulates EZH2 (log2FC = -0.6; FDR = 0.01) and

| Table 4 | Seven epigenetic modifiers that bind TINCR |
|---------|------------------------------------------|
| Gene ID | Name | Log2FC HIR/LIR | FDR HIR/LIR | Log2FC HIR/ GnRHa | FDR HIR/ GnRHa |
| SETD7 | SET domain containing lysine methyltransferase 7 | + 0.22 | 0.042 | -0.85 | 0.0006 |
| ARID4B | AT-rich interaction domain 4B | + 0.18 | 0.041 | -0.63 | 0.008 |
| ARID5B | AT-rich interaction domain 5B | + 0.35 | 0.004 | -0.49 | 0.003 |
| KDM6A | lysine demethylase 6A | + 0.20 | 0.017 | -0.81 | 0.0009 |
| CHD6 | chromodomain helicase DNA binding protein 6 | + 0.21 | 0.040 | -0.54 | 0.03 |
| MBD2 | methyl-CpG binding domain protein 2 | + 0.23 | 0.029 | -0.68 | 0.004 |
| BPTF | bromodomain PHD finger transcription factor | + 0.19 | 0.055 | -0.61 | 0.01 |

The log-fold changes (FC), p-value, false discovery rate (FDR), comparing HIR and LIR cryptorchid testes as well as results from HIR group following GnRHa treatment are presented.
implicates the regulation of *HOTTIP* gene transcription, which then transcriptionally regulates the HOXA cluster. As a result, an increase occurs in *HOXA2* (log2FC = 2.38; FDR = 0.007), *HOXA3* (log2FC = 1.68; FDR = 0.007), *HOXA11* (log2FC = 1.77; FDR = 0.02), and *HOXA-AS3* (log2FC = 2.68; FDR = 0.0001).

Gamma-aminobutyric acid (GABA) plays a key developmental role in the regulation of GnRH neuron migration from the olfactory placodes into the forebrain during fetal development [52], and co-expression of *DLX3* and *PAX6* proteins, correlates with acquisition of the olfactory placode fate [53]. Moreover, GABA-A receptors and GABA transporter 1 (*GAT1*) have been reported to be involved in the proliferation of Leydig cells, testosterone production, and spermatogenesis [54]. GABA3 (log2FC = −2.49; FDR = 0.0001) and GABR5 (log2FC = −2.59; FDR = 0.0006) were downregulated in HIR tests following GnRHa treatment, an increase expression was observed in *DLX3*, *PAX6* [12], and *TP63* (log2FC = 0.91; FDR = 0.002), whereby the latter was downregulated in HIR (log2FC = −1.58; FDR = 0.001). Berghoff et al. proposed a model in which DLX6-AS1 inhibits the ultraconserved DNA methylation mark in *DLX5/6*, facilitating antagonistic interactions between repressive and activating transcription factors *MECP2* and *DLX* [53]. *DLX2*, *DLX3*, *DLX5* and *DLX6* showed low or no expression in HIR samples [12]. Loss of *DLX1/2* increases site-specific methylation of the *DLX5/6* ultraconserved enhancer [53]. *Tp63* regulates *DLX5* and *DLX6* transcription, at least in part, via cis-acting regulation at the promoter level [55]. These interactions allow differential control of adjacent genes by shared DNA regulatory elements [56]. It was also shown that deletion of *Dlx5* and *Dlx6* in the mouse leads to decreased testosterone levels and an abnormal masculinization phenotype [42]. Thus, impaired steroidogenesis during mini-puberty in HIR boys may be induced by altered GABA-A receptor signaling, silenced *DLX6* expression as well as destabilization of AR and ERs.

GnRHa treatment affects genes associated with normal and pathological cell division

Several recent studies have reported roles for IncRNAs in cancer, including *MALAT1* and GAS, strongly indicating that IncRNAs not only control gene regulatory pathways in normal cells and tissue, but also during tumor development [57, 58]. The occurrence and progression of cancer is the result of a combination of multiple factors. Furthermore, cancer-specific IncRNA expression patterns appear to be more tissue- and stage-specific than those of protein-coding genes, supporting the potential development of IncRNAs as efficient biomarkers and therapeutic targets [58]. Interestingly, GnRHa treatment increased LH and testosterone secretion and *HOTAIR* expression, and probably its recruitment to chromatin, whereas the expression of *MALAT1* was reduced (log2FC = −0.64; FDR = 0.03; REF), suggesting opposite regulation and functions of these IncRNAs during normal testis development. It was reported that LINC-ROR promote liver cancer stem cell growth by upregulating *TERT* and *C-MYC* [59]. Notably, GnRHa treatment stimulates the expression of *TERT* (log2FC = 0.91; FDR = 0.002; [46]), which is decreased in HIR (log2FC = −1.58; FDR = 0.001;) [46] but downregulates *C-MYC* signaling (log2FC = −0.58; FDR = 0.01;).

Variation in lincRNA expression may be associated with cancer progression. For example, *LINC00261*, which plays an important role in gastric cancer, is stimulated by GnRHa, and exerts tumor suppressive activity by reducing cancer cell invasion via suppression of the epithelial-mesenchymal transition process [60]. Another lincRNA, *TINCR*, is involved in normal tissue differentiation and plays a critical role in cancer and metastasis. *TINCR* expression is downregulated in HIR (log2FC = −1.07; FDR = 0.002) [12]. We observed that *TINCR* depletion in HIR tests resulted in the induction of key epigenetic modifiers, seven of which were downregulated following GnRHa treatment (Table 4). One of them, *SETD7*, is an epigenetic modifier and regulates AR [61]. *SETD7* binds *TINCR* and may mediate the formation of AR-associated coactivator complexes. Taken together, the results indicate that the HIR group of cryptorchid boys with abortive mini-puberty expresses several cancer genes. Gonadotropin-releasing hormone treatment may protect against testicular tumor development by downregulating oncogenes, such as *MALAT1*, *mTOR*, *C-MYC*, and *EZH2* to enable normal germ cell development.

Conclusions

Two major goals in the field of male reproductive biology are to elucidate the molecular mechanisms that underlie cryptorchidism and to develop an effective treatment for its long-term consequences. According to the mini-puberty hypothesis, early-life exposure to gonadal hormones during a specific window of sensitivity triggers sex-specific developmental processes. To preserve molecular features of differentiated cells, it is crucial that transcriptional alterations triggered by external or intrinsic signals continue beyond the initial stimulus. One possible mechanism involves epigenetic histone variant replacement, chromatin remodeling factors, and IncRNAs associated with epigenetic factors. We found that many of the lincRNAs responding to GnRHa treatment are associated with somatic cancer. This may reflect the fact that the hormone stimulates germ stem cell growth and Leydig, as well as Sertoli, cell division. The mechanisms involved are likely rather diverse and may include promoter/enhancer activity, miRNA sponge
activity, or control of gene expression via RNA-RNA interactions. We propose that the IncRNAs identified in this study may be involved in establishing normal male fertility by acting at early stages of spermatogonia stem cell development and by affecting other testicular cells capable of responding to GnRH treatment. Our results provide information for further functional analysis of long-noncoding RNA in relation to the infertility development. We propose that the HOTAIR and DLX pathways, as well as both canonical and non-canonical WNT pathways, are involved in Ad spermatogonia growth and differentiation.

Abbreviations

1PRDM6: PR/SET domain 6; ARN: Antisense of IGF2R (insulin like growth factor 2 receptor) non-protein coding RNA; AR: Androgen receptor; ARD4B: AT-rich interaction domain 4B; ARDSB: AT-rich interaction domain 5B; AS-InC-2: Antisense long non-coding RNA; BMP4: Bone morphogenetic protein 4; BMP5: Bone morphogenetic protein 5; BOD1L2: Biorecognition of chromosomes in cell division 1 like 2; BPTF: Bromodomain PHD finger transcription factor; CDH11: Cadherin 11; CDH5: Cadherin 5; CDH6: Cadherin 6; C-MYC: v-myc avian myelocytomatosis viral oncogene homolog; CXC1: Chemokine; DBD: DNA-binding domain; dbGaP: Database of Genotypes and Phenotypes; DCBLD2: Discoidin, CUB and LCC domain containing 2; DLX: Distal-less homeobox; DLX2: Distal-less homeobox 2 (Homo sapiens [human]); DLX3: Distal-less homeobox 3 (Homo sapiens [human]); DLX5: Distal-less homeobox 5 (Mus musculus [mouse]); DLX6: Distal-less homeobox 6 (Mus musculus [mouse]); DLX6-A51: Distal-less homeobox 6 antisense RNA 1; DMRTC2: DMRT (dovetail and marb-3 related transcription factor) like family C2; EZ: Estradiol; EGF-R: Epidermal growth factor receptor; EGFR-A51: Epidermal growth factor receptor antisense RNA 1; ERS: Estrogen response elements; ERIC-A51: Glutamate rich protein antisense RNA 1; ERs: Estrogen receptors; ESC: Embryonic stem-cell; EZH2: Enhancer of zeste 2 polycomb repressive complex 2 subunit; FDR: False discovery rate; FENDRR: FOXF1 (forkhead box F1) adjacent non-coding developmental regulatory RNA; FOXA1: Forkhead box A1; FOXA2: Forkhead box A2; FSH: Follicle stimulating hormone; GABA: Gamma-aminobutyric acid; GABA-A: Gamma-aminobutyric acid type A; GABRA-A: gamma-aminobutyric acid (GABA) A receptor, alpha 1; GABRA-A5: Gamma-aminobutyric acid (GABRA) A receptor 5; GARP: Golgi-associated retrograde protein; GAS: Growth arrest specific; GAT1: GABA (gamma-aminobutyric acid) transporter 1; GDNF: GDNF family member 4; GDNF-like: Glial cell derived neurotrophic factor; GnRHa: Gonadotropin releasing hormone antagonist; H3K27: Histone H3 lysine 27; H3K4: Histone H3 lysine 4; HAGLR: HOXD (homeobox D cluster) antisense long non-protein coding RNA; HIR: High infertility risk; HOTAIR: HOX (homeobox) transcript antisense RNA; HOTITP: HOXA (mouse) X cluster distal transcript antisense RNA; HOXA11: Homeobox A11; HOXA13: Homeobox A13; HOXAX: Homeobox A2; HOXAX3: Homeobox A3; HOXAX9: Homeobox A9; HOXAX3-A33: Homeobox A9. (Homeobox A cluster) antisense RNA 3; KDM6A: Lysine demethylase 6A; LH: Luteinizing hormone; LINC01553: Long intergenic non-protein coding RNA 1553; LINE1-A: Long non-protein coding RNA; LINC02249: Long intergenic non-protein coding RNA 249; LINC01446: Long intergenic non-protein coding RNA 1446; LINC01553: Long intergenic non-protein coding RNA 1553; lincRNA-A: Long non-protein coding RNA; Lnc-ROR: Long intergenic non-protein coding RNA, regulator of reprogramming; LIR: Low infertility risk; IncRNA: Long non-coding RNA; log2FC: log2 fold change; MALAT1: Metastasis associated lung adenocarcinoma transcript 1; MBD2: Methyl-CpG binding domain protein 2; MECP2: Methyl-CpG binding protein 2; mir-138: microRNA 138; mir-145: microRNA 1; mir-489-3p: microRNA 489; MLN: Motilin; MTOR: mechanistic target of rapamycin kinase; MTOR-A51: MTOR (mechanistic target of rapamycin kinase) antisense RNA 1; NAT RNA: N-acetyltransferase RNA; NATs: Natural antisense transcripts; NEU3: Neuraminidase 3; OSR1: Odd-skipped related transcription factor 1; OTX1: Orthodenticle homeobox 1; OTX2: Orthodenticle homeobox 2; OTX2-A51: Orthodenticle homeobox 2 antisense RNA 1 (head to head); PAI: Paired box 1; PAIX6: Paired box 6; PAIX7: Paired box 7; P3K/Akt pathway: Phosphatidylinositold 3-phosphate kinase, putative, and protein kinase B pathway; PRD1: PR domain containing 1, with ZNF domain; PRD12: PR/SET domain 12; PRD14: PR/SET domain 14; PRD16: PR/SET domain 16; PRD17: PR/SET domain 17; PRDM9: PR/SET domain 9; PRDM13: PR/SET domain 13; PSMOD6: Proteasome 26S subunit, non-ATPase (adenosine triphosphatase) 6; RPKM: Reads per kilo base per million mapped reads; S/ T: Total germ cell count per tubule; SETD7: SET domain containing 7, histone lysine methyltransferase; SLC2: Solute carrier family 25 member 37; SOX11: SRY (sex determining region Y)-box transcription factor 11; SOX13: SRY (sex determining region Y)-box transcription factor 13; SQX4: SRY (sex determining region Y)-box transcription factor 4; SPIDR: Scaffolding protein involved in DNA repair; SRY: Sex determining region of Y; T: Brachury; TCF7L2: Transcription factor 7 like 2; TERT: Telomerase reverse transcriptase; TIMELESS: Timeless circadian regulator; TINCR: TINCR (Tissue differentiation-inducing non-protein coding RNA) ubiquitin domain containing, TIP63: Tumor protein p63; TPT1: Tumor protein, translationally-controlled 1; TTVT: Transcripts specific transcript, Y-linked; U1P1: Ubiquitin specific peptidase 1; VPS53: VPS53 (Vacuolar protein sorting homolog 3) subunit of GARP (Golgi-associated retrograde protein) complex; Wnt: Wingless-related integration site; WNT3: Wnt family member 3; WNT4: Wnt family member 4; XIST: Inactive X specific transcripts.

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Authors’ contributions

FH conceived and designed the study, interpreted the data, and organized and wrote the manuscript. GV performed experiments, analyzed the data, and read the paper. BV performed experiments and read the paper. MBS analyzed and interpreted the data, contributed analysis tools, and read the paper.

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Availability of data and materials

Raw data files were deposited at the Database of Genotypes and Phenotypes (dbGaP) under accession number phs001275.v1.p1.

Ethics approval and consent to participate

Investigations were carried out in accordance with the Declaration of Helsinki of 1975 (revised in 2008). The study was approved by the Institutional Review Board and the Independent Ethics Committee of Vilnius University (Vilnius Regional Biomedical Research Ethics Committee, No. 135200–580-PP1-17, 11 June 2013).

Consent for publication

Written informed consent was obtained from the patients’ guardians after approval by the ethical committee.

Competing interests

The authors declare no conflicts of interest.

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