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EGR4 Is a Master Gene Responsible for Fertility in Cryptorchidism

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Cryptorchidism · Infertility · Ad spermatogonia · EGR4 · Microarray

Abstract
The purpose of early medical or surgical treatment of boys with undescended testes is to prevent the development of infertility. However, early and successful surgery cannot prevent infertility in cryptorchid boys who lack type A dark (Ad) spermatogonia. The aim of this study was to compare the gene expression pattern of patients with completed transformation of gonocytes into Ad spermatogonia, associated with low infertility risk, with patients that had failed to undergo this process and had a high infertility risk. Genes expressed in the 16 cryptorchid testes were estimated using Affymetrix whole-genome microarray and compared to the expression profiles from four contralateral gonads of boys with unilateral testicular agenesis. Whole-genome expression profiling showed that boys in the high infertility risk group according to testicular histology, showed decreased or lack of expression of most of the genes essential for hypothalamo-pituitary-testicular axis function relative to low or intermediate risk group as well as controls. In particular, *EGR4*, which is involved in regulating the secretion of luteinizing hormone, was virtually not expressed. Thus, we found multiple differences in gene expression between the high and low infertility risk groups, confirming the importance of an intact hypothalamo-pituitary testicular axis and *EGR4* in fertility development.

Undescended testis (cryptorchidism) is the most common genitourinary disorder of childhood, resulting in 27,000 surgeries each year in the United States [Trussell and Lee, 2004]. The hallmark of successful treatment of cryptorchid boys is fertility. Over the last 40 years, histological studies have improved our understanding of the etiology of cryptorchidism. Leydig cell atrophy found already in infancy, increased collagenisation of peritubular connective tissue, and age-related loss of germ cells resulted in therapeutic shift from 8 years of age into the first year of life. Type A dark (Ad) spermatogonia have been identified as a key marker of potential fertility: 94% of men who had Ad spermatogonia in biopsies taken at the time of orchidopexy had normal sperm counts; in contrast, 92% of men without Ad spermatogonia at the time of orchidopexy showed abnormal spermiograms [Hadziselimovic and Herzog, 2001; Hadziselimovic et al., 2007]. Thus, differences in sperm production after early and successful orchidopexy could be predicted by the

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presence or absence of Ad spermatogonia. Comparisons of histology and hormone levels have revealed that hypogonadotropic hypogonadism is present in a majority of cryptorchid boys [Hadziselimovic and Hoecht, 2008]. Microarray profiling of germ cell development and differentiation has established a connection between DNA sequence data and protein function and interactions.

Our objective was to compare and understand expression program differences between groups of patients with cryptorchidism, particularly, those with completed transformation of gonocytes into Ad spermatogonia and those who had failed to undergo this process and developed infertility, despite successful orchidopexy. We used an Affymetrix whole-genome microarray that covers all currently annotated human genes.

Patients and Methods

Testicular Biopsies and Pooling of Patients

In our institutions, it is routine practice to perform testicular biopsy during surgery for undescended testis (orchidopexy). We have found that testicular biopsy provides useful information about future semen quality and helps to identify patients with atypical spermatogonia or carcinoma in situ. Furthermore, histological examination of the biopsies is the only way to obtain the diagnostic and prognostic information needed to establish the appropriate medical strategy for adults [Schulze et al., 1999] and children [Hadziselimovic and Hoecht, 2008].

Twenty testicular biopsies as large as the size of a kernel of rice from 19 boys were analyzed. From December 2007 until March 2008 all patients aged 10 months to 5 years having orchidopexy were included in the study. Sixteen testes from 15 boys with cryptorchidism were divided into three groups according to the presence or absence of Ad spermatogonia, and the total number of germ cells per tubule. Group 1 (n = 5) included the high infertility risk group (HIR) which had no Ad spermatogonia and a total germ cell count per tubule of less than 0.2; group 2 (n = 5) included the intermittent infertility risk group (IIR) which had a total germ cell count of >0.2, but <0.6 germ cell per tubule, and an Ad spermatogonia count of <0.005 per tubule. Group 3 (n = 6) included the low infertility risk group (LIR) which had >0.1 Ad spermatogonia/tubule and more than 1 germ cell per tubule. The contralateral descended testes from patients with testicular agenesis have been shown to have more Leydig and germ cells and a higher rate of transformation of Ad spermatogonia into spermatocytes than the contralateral descended testes of patients with unilateral cryptorchidism [Huff et al., 1991]. As not all contralateral testes are expected to have normal histology and may require subsequent hormonal treatment we routinely biopsy these testes during the period of testicular fixation to prevent testicular torsion. Four testes judged post-hoc to have an identical histology as normal prepubertal testis served as a 'control' group [Seguchi and Hadziselimovic, 1974; Paniagua and Niestal, 1984]. The age of the patients at surgery was: HIR: $\bar{x}$: 2.9 (95% CI 0.5–5.84), LIR: $\bar{x}$: 3.6 (95% CI 2.78–4.41), IIR: $\bar{x}$: 3.12 (95% CI 1.69–4.51), control: $\bar{x}$: 3.82 (95% CI 2.29–5.35) years. All patients had an extensive clinical examination and we could not find any clinical signs of developmental malformations or syndromes. In particular no sign for Kallmann syndrome was observed. Furthermore, all patients had normal thyroid screening and no features of hypopituitarism were discernible. Testicular position at surgery was: HIR: one abdominal, three at tubercle, LIR: one inguinal, five at tubercle, IIR: one abdominal, three at tubercle, one inguinal. One patient from HIR and one from LIR had hormonal treatment with LH-RH 5 months prior to the surgery. Biopsies were fixed in 3% glutaraldehyde and embedded in Epon. Semi-thin sections, 1-μm thick, were examined by Zeiss Axioscope phase contrast and conventional light microscope, 63×: 1.40 oil: plan-apochromat. Photographs were performed with a Canon power-shot camera. For each biopsy, at least 200 tubular cross sections were evaluated. Immediately following biopsy, one-half of each biopsy sample was collected for RNA isolation and GeneChip hybridization and was stored in cold 1× phosphate buffered saline (PBS). The tissue was then filtered through 100-nm nylon gauze. Fractions were collected, washed three times in 1× PBS, and snap-frozen in liquid nitrogen prior to storage at –80°C. An aliquot of each fraction was fixed in 18× PBS containing 1% formaldehyde (Polio, France) and 1% fetal calf serum (Eurabbie, France) and analyzed by DNA flow cytometry to determine its relative DNA content. Cell pellets and tissues were sheared in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol and further processed using a Qiashredder (Qiagen). The suspension was centrifuged for 2 min at maximum speed and the clarified lysate was stored at –80°C. Total RNA isolation was performed using RNeasy Mini-Spin columns (Qiagen) according to standard protocols. RNA quality was monitored using RNA Nano 6000 Chips and the 2100 BioAnalyzer (Agilent). cRNA synthesis, human U133 plus 2.0 GeneChip hybridization, and raw data recovery were carried out as published previously [Chalmel et al., 2007]. Raw data files are available at the EBI ArrayExpress repository [Parkinson et al., 2007] via the accession numbers E-TABM-130 and E-TABM-174 and GEO [GSE16191].

Statistical Analysis and Interpretation of Microarray Data

The data analysis and gene filtering was performed using R/Bioconductor [Gentleman et al., 2004]. Signal condensation was performed using only the RMA from the Bioconductor Affy package. Differentially expressed genes were indentified using the empirical Bayes method (F-test) implemented in the LIMMA package and adjusted with the False Discovery Rate (FDR) method [Wettenhall and Smyth, 2004]. We selected those probe sets with a log2 average contrast signal of at least 5, an adjusted p value <0.05 and an absolute log2 fold change of >0.585 (1.5-fold in linear space). Hierarchical clustering and visualization was performed in R. Gene lists from the resulting contrasts were further classified into regulatory networks and pathways with Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid into a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Functional analyses associated with biological

Table 1. Primers used in this study

Genes	Forward primer	Reverse primer
TFRC1	TCGTCCCTGCATTTAAAGGCTTTC	CAACAGTGGGCTGGCAGAAAC
GAPDH	GATCATCAGCAATGCCTCCTG	GAGTCCTTCCACGATACCAAAG
EGR4	AGCAAGAGATGGGTTTATG	AGGAGTTGGAAGAAGAGC
DLX2	TTAGGTGCCTTTGCGGATGAC	ACAAACTCTGTGTCCAAGTCCAG
DDX25	AGAATGTCTCAGTGGGTTTTG	GCTTCACATCAATTTGGCTTC
MAGEA4	GGCCAGTGCATCTAACAG	AAATAGAAACCCACTACTAAGAAC

functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered, and Fisher's exact test was used to calculate a p value determining the probability that each biological function and/or disease assigned to that data set is due to chance and chance alone. The networks/pathways shown depict a graphical representation of the molecular relationships between genes/gene products.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed using a Corbett Research RG-6000 instrument. cDNAs were synthesized with the Reverse Transcriptase Core Kit (Eurogentec, cat RT-RTCK-03) using random primers (table 1). Real-time PCR runs were performed using SyBr Fast Kit (Kapa Biosystems, Cat# KK4602) with each gene-specific primer at 200 nM final concentration in a total volume of 17.5 μl. Length waves of source and detection were set at 470 nm and 510 nm, respectively. Gain was set at 8.33. PCR program was set as follows: 95°C, 60 s – 45 times (95°C, 3 s – 60°C, 10 s – 72°C, 4 s) followed by a melting curve analysis (65°C to 95°C, rising by 0.65°C each step) to attest amplification specificity. Threshold cycles (crossing point) were determined using Rotor-Gene software version 6.1. Good PCR efficiency was checked by performing a dilution series of the cDNA. Minus RT controls were performed for each sample studied. Threshold cycles (crossing point) were determined using Rotor-Gene software version 6.1. Expression levels were normalized to TFRC1 and GAPDH using a geometric mean of their level of expression. Those genes were selected because they showed minimum variation between individual samples (both on microarrays and by qPCR). Fold differences were calculated using the delta-delta Ct method (fig. 1).

Antibody Validation

For immunohistochemical analysis, Epon was removed from the tissue sections. The sections were treated with 2% bovine serum albumin to reduce non-specific binding and then incubated with primary antibody overnight at 4°C. All samples were washed with PBS between incubations. We validated nine antibodies: DDX25 [SC51269/A2108], EGR1 [AB-54966/509443], EGR4 [SC-19868/JU306], CBL [SC-1651/80206], ACVR1B1 [AB-71539/603652], ALDH1A2 [SC-22591/1008], KLF4 [AB-26648/64129], MAGEA4 [SC-28484/62006], COL4A3 [SC-18177/L1404]. Secondary antibodies, labeled with polymer-horseradish peroxidase [HRP; goat polyclonal anti-rabbit IgG, mouse IgG and IgM (prediluted; ab2891); Abcam, Cambridge, UK] were used to detect binding of the primary antibody. The chromogenic reaction was developed by adding a freshly prepared solution of 3,3-diaminobenzidine solution (DAB + chromogen; DAKO). The DAB reaction was terminated by washing in TRIS-buffered saline (TBS 0.05 M and 0.85 M NaCl, pH 7.6). To visualize the histology of testicular cells, the samples were counterstained with toluidine blue. Antibody binding was indicated by a brown precipitate. Different cell types were identified based on their nuclear morphology and position within the developing gonad. Immunohistochemistry experiments were performed at least twice on at least four patients from each group, and only those with identical results between experiments for each sample were considered acceptable. Controls for non-specific binding of the secondary antibody were performed in all experiments by omitting the primary antibody; these consistently yielded no signal within the seminiferous epithelium or the interstitial space. The interstitial staining observed in the presence of the primary antibody was considered to be non-specific because it was not associated with, or localized within, a particular cell type. However, staining of interstitial cells was recorded. Experimental design, biomaterials and treatments, reporters, staining, imaging data, and image characterization were performed in compliance with the minimum information specification for immunohistochemistry experiments [Deutsch et al., 2008].

Ethical Considerations and Approval

In accordance with the Declaration of Helsinki, the Institutional Review Board and the Independent Ethics Committee of the University Children's Hospital Würzburg and the Children's Clinic Liestal approved all aspects of this study. Approval was provided for research involving the use of material (data records or specimens) that had originally been collected for non-research purposes.

Results

Total RNA and cRNA samples were of high quality, the signal intensity distributions were similar and were within the normal range. As expected, overall signal intensities visualized using a distance matrix grouped HIR, IIR, LIR and control patients into separate groups (fig. 2). Of the transcripts overlapping the four groups, 432 transcripts were differentially expressed in HIR and LIR tes-

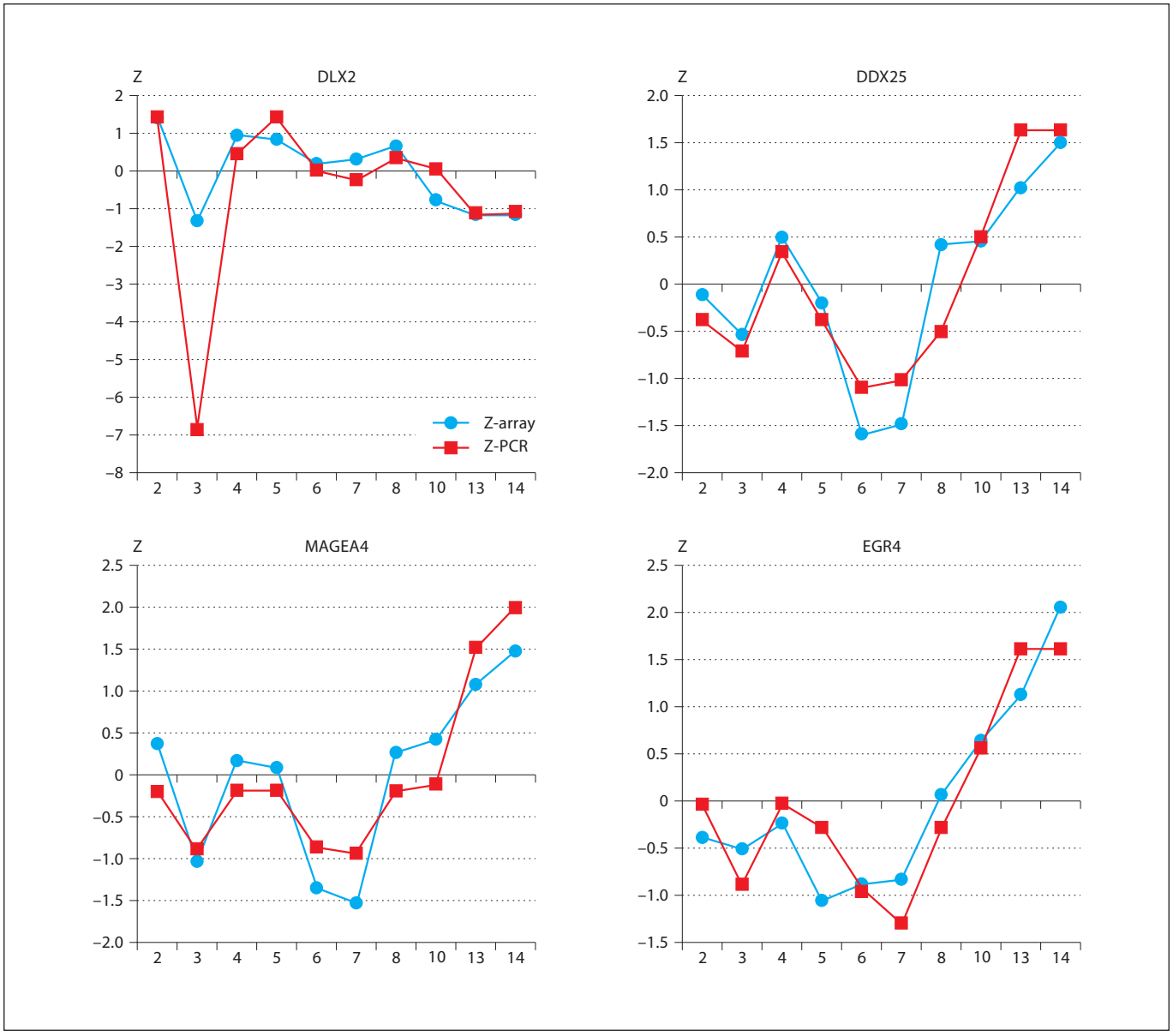

Fig. 1. Correlation of microarray data with data obtained by qRT-PCR on four genes that displayed significant expression changes, showing a high correlation in the obtained transcriptional profiles. The y-axis is Z-normalized log2 expression (array) and 2-normalized QT value (QPCR). The x-axis shows biopsy file names.

tes after statistical filtering. This gene list comprised 34 elevated, and 398 decreased transcripts in HIR testes. In HIR and IIR testes, 578 transcripts were differentially expressed; 527 down-regulated and 51 up-regulated. We found that 5 genes (one gene with unknown function, *PLN, TAGLN, C4A,* and *RERGL*) were down-regulated in the LIR patients in comparison to the IIR group. Eight genes were up-regulated (four genes with unknown function, *HBG1, FAM169A, LOC64450* and *CYP26B1*) in the LIR, compared to the IIR group. Except for *CYP26B1*, none of the genes that were differentially expressed were directly involved in the hormonal control of the reproductive axis.

We next investigated if and how the expressed genes were associated with the reproductive axis and found that the majority of the differentially expressed genes involved

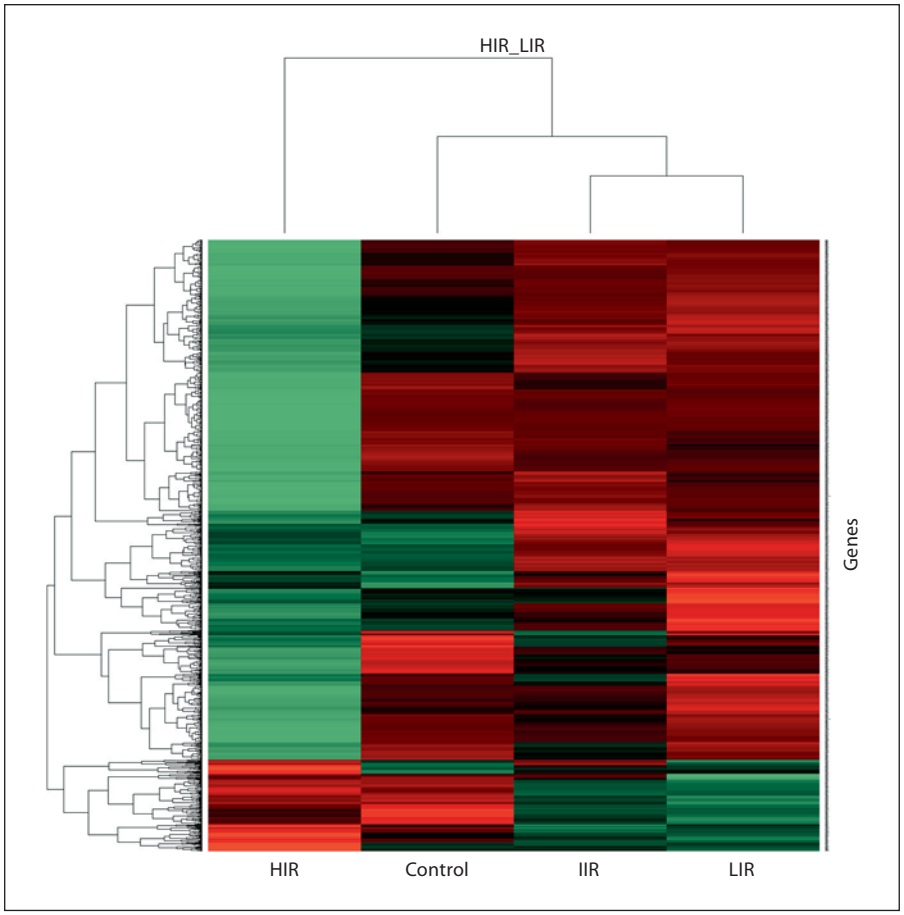

Fig. 2. Heat map (unsupervised) showing hierarchical clustering of gene expression profiles.

in the hormonal control of the reproductive axis were expressed to lesser extents in the HIR group (table 2, fig. 3). Despite identical age at surgery and testicular position, patients in the HIR group had virtually no *EGR4* expression in contrast to the other three groups (table 2, fig. 4). Both the IIR and the LIR groups expressed *EGR4* in the same way as the control group (table 2, fig. 4).

Further downstream in the LH pathway, *DDX25* (which is gonadotropin and androgen dependent) was expressed less in the HIR group than in the control and LIR group (table 2). The following key regulators of spermatogenesis were expressed to a lesser extent in the HIR group than in the LIR, IIR and control groups: *CREM*, *DAZL*, *PIWIL2*, *RHOXF1*, *RNF17*, *TAF7L*, *TEX14*, furthermore, cancer testis associated proteins (CTAs); MAGE, GAGE, SSX as well as *ALDH1* and *CBL*, genes involved in testosterone synthesis and regulation (table 2). Different FSH-dependent genes, such as *ADA, FGFR3, FST, ID4, NRG1* as well as genes associated with GnRH control, including *FOXG1, DLX5, LHX6, ISL1,* and *NMU* were underexpressed in the HIR group (table 2). The expression signature of WNT family genes, which activate a canonical WNT-β-catenin pathway, was equivalent in all groups, except for *WNT3*, which was absent in the HIR group (table 2). Nevertheless, *GNRH1* and *GNRH2* were expressed equally in all groups, (data not shown). At testicular level both LHB and FSHB expression were not significantly different among groups studied; [LHB (HIR) 5.36 (95% CI 4.97–5.75); LHB (LIR) 5.58 (95% CI 5.36–5.81); FSHB (HIR) 4.01 (95% CI 3.79–4.24); FSHB (LIR) 3.87 (95% CI 3.65–4.09)].

Immunohistochemical Validation

We wanted to determine whether greater transcript expression corresponded to greater protein expression. Immunoperoxidase histochemistry (IHC), although not a quantitative method, is currently the only practical way to detect proteins in small fixed tissue samples. Employing several antibodies to proteins known to be involved in the reproductive hormonal axis, we compared histo-

Table 2. Genes involved in the hypothalamo-pituitary-testicular axis down-regulated in HIR. Gene ID, name and gene function are indicated. p values between groups analyzed are presented. Gene expression in Leydig or germ cells according to ArrayExpress (Atlas of gene expression, EMBL Heidelberg) was declared with yes, no or n.f. for each gene studied

Gene ID	Name	Function	Leydig cell	Germ cell	HIR/Co p value	LIR/Co p value	IIR/Co p value	HIR/LIR p value	HIR/IIR p value	LIR/IIR p value
ADA	adenosine deaminase	Sertoli cell developmental factor	n.f.	n.f.	3.72E–08	n.s.	n.s.	1.38E–06	0.0009	n.s.
ALDH1	aldehyde dehydrogenase	participates in the oxidation of all-trans retinal to retinoic acid	yes	yes	0.0009	n.s.	n.s.	0.0033	0.002	n.s.
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	regulates androgen-dependent apoptosis in spermatogonia	yes	yes	1.46E–05	n.s.	n.s.	2.23E–06	7.50E–05	n.s.
CREM	cAMP responsive element modulator	involved in mammalian spermatogenesis and spermiogenesis	yes	yes	5.21E–05	n.s.	n.s.	5.47E–05	0.0001	n.s.
DAZL	deleted in azoospermia-like	found in azoospermia and Sertoli cells only	yes	yes	0.002	n.s.	n.s.	1.53E–02	0.036	n.s.
DDX25	DEAD (Asp-Glu-Ala-Asp) box polypeptide 25	essential for spermatogenesis and spermatid development	yes	yes	0.009	n.s.	n.s.	3.20E–02	n.s.	n.s.
DLX5	distal-less homeobox 5	development of the olfactory pathway	yes	yes	n.s.	n.s.	n.s.	n.s.	0.009	n.s.
EGR4	early growth response protein 4	master LH secretory gene	yes	yes	3.82E–06	n.s.	n.s.	2.34E–07	8.06E–07	n.s.
FGF9	fibroblast growth factor 9 (glia-activating factor)	gonadal development, germ cell maturation	n.f.	n.f.	n.s.	n.s.	n.s.	1.24E–05	0.0001	n.s.
FGFR3	fibroblast growth factor receptor 3	proliferation and differentiation of Sertoli cells	yes	yes	5.61E–07	n.s.	n.s.	2.50E–08	2.21E–07	n.s.
FOXG1	forkhead box G1	neuroendocrine regulation/ androgen receptor brain area	yes	yes	5.30E–05	n.s.	n.s.	1.00E–04	0.0001	n.s.
FST	follistatin	regulation of FSH secretion (embryo)	yes	yes	8.99E–05	n.s.	n.s.	3.06E–05	1.45E–05	n.s.
GAGE1	G antigen, 1	cancer/testis antigens (CTAs), germ cell development	yes	yes	4.15E–07	n.s.	n.s.	4.03E–06	4.54E–06	n.s.
GAGE3	G antigen, 3	proliferation, differentiation and survival of germ cells	yes	yes	4.71E–07	n.s.	n.s.	2.23E–05	7.04E+06	n.s.
GAGE12B	G antigen, 12B	proliferation, differentiation and survival of germ cells	yes	yes	1.10E–06	n.s.	n.s.	8.04E–06	4.54E–06	n.s.
GAGE12F	G antigen, 12F	proliferation, differentiation and survival of germ cells	n.f.	n.f.	6.18E–07	n.s.	n.s.	8.04E–06	2.41E–06	n.s.
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	Id4 differentiation and hormone regulation of Sertoli cells	yes	yes	6.19E–05	n.s.	n.s.	4.30E–04	0.0004	n.s.
ISL1	ISL LIM homeobox 1	LIM homeodomain factor	n.f.	n.f.	1.03E–05	n.s.	n.s.	4.23E–07	5.19E–06	n.s.
LHX6	LIM homeobox 6	pituitary development	yes	yes	n.s.	n.s.	n.s.	2.00E–04	0.0007	n.s.
MAGEA1	melanoma antigen family A, 1 (directs expression of antigen MZ2-E)	proliferation, differentiation and survival of germ cells	yes	yes	5.76E–06	n.s.	n.s.	n.s.	6.11E–05	n.s.
MAGEA2	melanoma antigen family A, 2	proliferation, differentiation and survival of germ cells	n.f.	n.f.	4.41E–08	n.s.	n.s.	1.49E–07	3.03E–08	n.s.
MAGEA3	melanoma antigen family A, 3	proliferation, differentiation and survival of germ cells	n.f.	n.f.	3.97E–08	n.s.	n.s.	6.98E–08	3.03E–08	n.s.
MAGEA4	melanoma antigen family A, 4	proliferation, differentiation and survival of germ cells	yes	yes	2.26E+06	n.s.	n.s.	1.06E–05	3.08E–05	n.s.
MAGEA5	melanoma antigen family A, 5	proliferation, differentiation and survival of germ cells	n.f.	n.f.	7.20E–06	n.s.	n.s.	n.s.	7.00E–04	n.s.
MAGEA12	melanoma antigen family A, 12	proliferation, differentiation and survival of germ cells	n.f.	n.f.	5.76E–06	n.s.	n.s.	8.53E–05	6.03E–05	n.s.
MAGEB2	melanoma antigen family B, 2	proliferation, differentiation and survival of germ cells	yes	yes	2.62E–08	n.s.	n.s.	2.81E–09	1.04E–08	n.s.
MAGEC1	melanoma antigen family C, 1	proliferation, differentiation and survival of germ cells	yes	yes	4.87E–08	n.s.	n.s.	1.58E–09	1.94E–08	n.s.
NMU	neuromedin U	regulation of neuroendocrine gonadotropin axis	n.f.	n.f.	4.00E–04	n.s.	n.s.	4.45E–06	2.46E–05	n.s.

Table 2 (continued)

Gene ID	Name	Function	Leydig cell	Germ cell	HIR/Co p value	LIR/Co p value	IIR/Co p value	HIR/LIR p value	HIR/IIR p value	LIR/IIR p value
NRG1	neuregulin 1	proliferation of germ cells	yes	yes	1.00E–03	n.s.	n.s	1.06E–05	3.00E–04	n.s.
PIWIL2	piwi-like 2 (Drosophila)	regulation of spermatogenesis	yes	yes	2.27E–05	n.s.	n.s	5.52E–06	4.17E–05	n.s.
RHOXF1	Rhox homeobox family, member 1	X-linked homeobox gene	yes	yes	2.00E–04	n.s.	n.s	6.27E–05	1.00E–04	n.s.
RNF17	ring finger protein 17	involved in male meiosis, cell division, spermatogenesis	yes	yes	9.31E–05	0.04	n.s	1.00E–03	1.00E–02	n.s.
SSX1	synovial sarcoma X breakpoint, 1	proliferation, differentiation and survival of germ cells	n.f.	n.f.	5.10E–04	n.s.	n.s	7.00E–03	4.00E–03	n.s.
SSX3	synovial sarcoma X breakpoint, 3	proliferation, differentiation and survival of germ cells	yes	yes	3.50E–05	n.s.	n.s	2.40E–02	n.s.	n.s.
SSX4	synovial sarcoma X breakpoint, 4	proliferation, differentiation and survival of germ cells	n.f.	n.f.	9.00E–04	n.s.	n.s	n.s.	n.s.	n.s.
TAF7L	TAF7-like RNA polymerase II, TATA box binding protein	required for male stem germ cell proliferation and differentiation	yes	yes	1.00E–02	n.s.	n.s	n.s.	n.s.	n.s.
TEX14	testis expressed 14	required for intercellular bridges in vertebrate germ cells	yes	yes	4.00E–04	n.s.	n.s	0.001	6.00E–03	n.s.
WNT3	wingless-type MMTV integration site family, member 3	involved in canonical beta-catenin pathway	n.f.	n.f.	5.00E–04	n.s.	n.s	9.09E–07	2.82E–05	n.s.

n.f. = Not found; n.s. = not significant; Co = control.

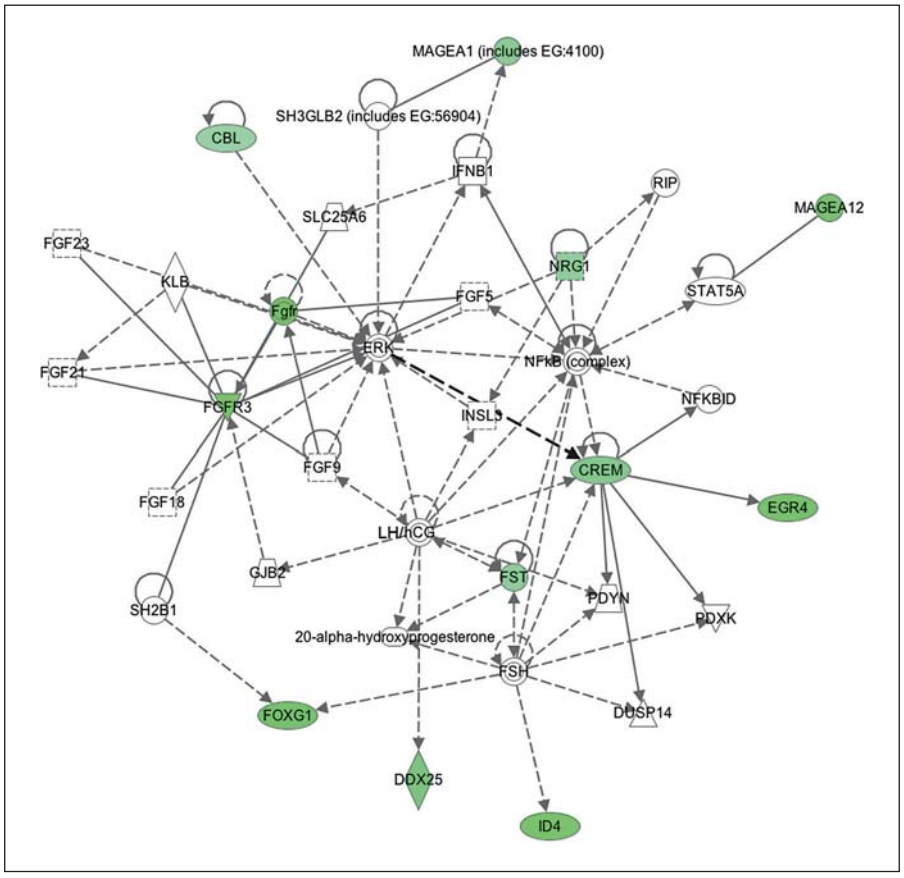

Fig. 3. In the HIR group, *EGR4* and 11 other genes are decreased in the LH/HCG network pathway (green). Focus genes showing graphical representations of the molecular relationship between genes. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The green node color indicates the degree of down-regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. (e.g. ◇ enzyme, □ cytokine, ○ other, ▽ kinase, □ (broken line): growth factor, ellipse: transcription factor, trapezium: transporter, △ phosphatase).

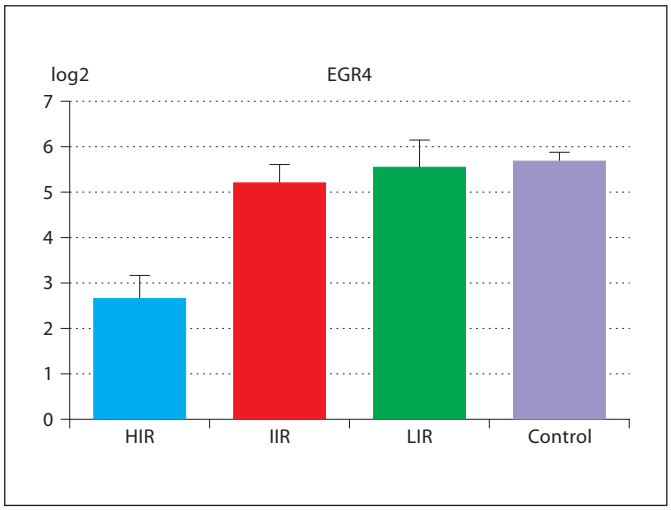

Fig. 4. *EGR4*, the master gene for fertility, is significantly less expressed in the HIR group, in comparison to the LIR, IIR and the control group. Median and average absolute deviation from median are presented.

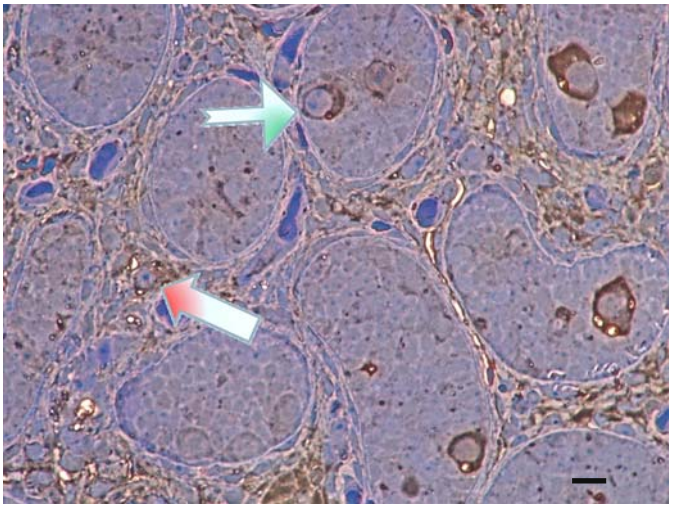

Fig. 6. Immunohistochemical staining of EGR4 in the LIR group testes showing EGR4 localized in the cytoplasm of Leydig (red arrow) and germ cells (green arrow). Contra staining with toluidine blue. (Horizontal bar; 0.1 mm).

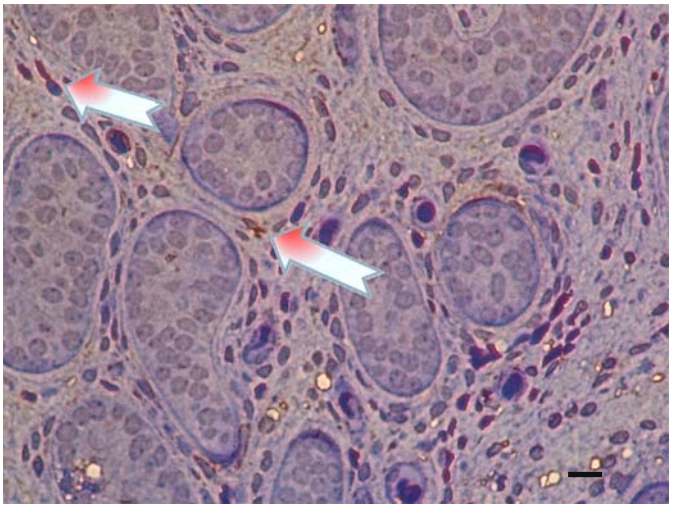

Fig. 5. Immunohistochemical validation of EGR4 in the HIR group. Atrophic Leydig cell cytoplasm was stained brown (arrow). Germ cells in the LIR group had the same expression as degenerating spermatogonia. Contra staining was performed with toluidine blue. There was no EGR4 in Sertoli cells and in the peritubular connective tissue. (Horizontal bar; 0.1 mm).

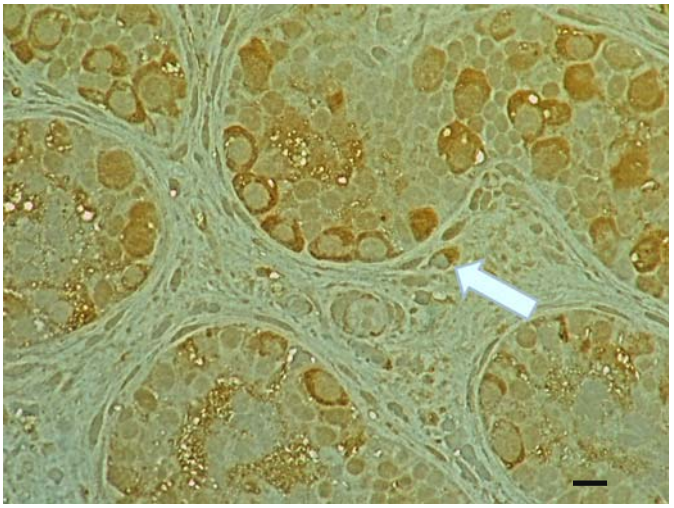

Fig. 7. Immunohistochemical validations at protein level for EGR4 in the control testes. Brown stained cytoplasm of Leydig cell (arrow) and the majority of spermatogonia. EGR4 was localized predominately in the cytoplasm and had translocated to the nucleus in some germ cells. (Horizontal bar; 0.1 mm).

logical sections of four histological subtypes. EGR4 protein was expressed in spermatogonia in all groups, while its expression in juvenile Leydig cells was more prominent in the LIR, IIR and control groups than in the HIR group (figs. 5–7). Surprisingly, most of the EGR4 protein was localized in the cytoplasm of the germ and Leydig cells (figs. 5–7). Identical cytoplasmic localization was found for all proteins analyzed. In addition to their expression in germ and Leydig cells, CBL was expressed in Sertoli cells.

We have additionally validated our microarray data by qRT-PCR on four genes that showed significant expression changes, of which all showed a high correlation in the obtained transcriptional profiles (fig. 1).

Discussion

During mini-puberty, a time between 30 and 90 days of postnatal life in male infants, the substantial increase in GnRH secretion induces an increase in gonadotropin and testosterone production. As a consequence, transformation of gonocytes into Ad spermatogonia takes place. Ad spermatogonia have a characteristic nuclear feature that distinguishes them from the other germ cells, e.g. fetal, transient, and Ap spermatogonia [Seguchi and Hadziselimovic, 1974; Paniagua and Niestal, 1984]. Boys with cryptorchidism who lack Ad spermatogonia have low basal and stimulated gonadotropin plasma values, compatible with hypogonadotropic hypogonadism [Hadziselimovic et al., 1979]. The relationship of Ad spermatogonia and fertility is of fundamental importance [Hadziselimovic et al., 2005]. If transformation of gonocytes into Ad spermatogonia fails during infancy, infertility is inevitable [Hadziselimovic and Herzog, 2001; Hadziselimovic et al., 2007; Kim et al., 2008]. This was first reported in 2001 [Hadziselimovic and Herzog, 2001] and has been reconfirmed by us [Hadziselimovic et al., 2007] and others [Kim et al., 2008]. Kim et al. analyzed the histology of testicular biopsies from patients with bilateral cryptorchidism and confirmed the prognostic importance of Ad spermatogonia for fertility [Kim et al., 2008]. Furthermore, there is strong evidence that cryptochid boys will develop infertility, despite early and successful orchidopexy [Hadziselimovic and Herzog, 2001; Hadziselimovic and Hoecht, 2008].

The major observation in the current work was that the early growth response gene *EGR4* was not expressed in boys in the HIR group (table 2), while *EGR1* was insignificantly differentially expressed among groups studied. Egr4 appears to regulate critical genes involved in early stages of meiosis and has a singularly important role in male murine fertility [Tourtellotte et al., 1999]. Male mice carrying mutant forms of *Egr1* and *Egr4* were infertile due to defective *Lhb* regulation [Tourtellotte et al., 2000]. Importantly, *Egr1/Egr4* double-mutant mice developed infertility, despite normal levels of Fsh [Tourtellotte et al., 2000]. Although Egr1 is important, Egr4 is critical as a redundant transcription factor required for sustaining male fertility when *Egr1* is mutated in the germ line [Tourtellotte et al., 2000]. Furthermore, administration of either HCG or testosterone to male mice with disruptions in both *Egr4* and *Egr1* completely restored atrophic androgen-dependent organs. *Egr1*, which is expressed preferentially in Leydig cells in the testis, was up-regulated 9.5-fold in *Egr4*-null testis [Tourtellotte et al., 2000]. Interestingly, another strain of *Egr1*-deficient mice was described as having low levels of Lhb in the pituitary and in the serum, Leydig cell atrophy, and was sterile [Topilko et al., 1998]. A single injection of LH in *Erg1* mutant male mice restored fertility in about one-half of the animals, suggesting that LH deficiency was the main cause of their sterility [Topilko et al., 1998]. In our study, *EGR1* was identically expressed in all three groups. We expected that expression of *EGR1* would have been increased in the HIR group, but the absence of the expected increase could be interpreted as relative EGR1 insufficiency. Patients in the IIR/LIR groups do have Ad spermatogonia, although the amounts are different (lower in IIR). No qualitative structural changes in Ad spermatogonia could be found between the groups, while the HIR group had no Ad spermatogonia. Based on results from our previous work [Zivkovic et al., 2006], we know that development of Ad spermatogonia is LH and T dependent. Since EGRs are pivotal for LH secretion, this is an indirect evidence that EGR4 is important for Ad formation and that the LH-T axis is involved in this developmental process. Patients belonging to the HIR group had a severely reduced *EGR4* expression and the same testicular histology as *Egr1/Egr4* double mutant infertile mice, with a severe tubular and Leydig cell atrophy. As in *Egr* mutant mice, treating boys with cryptorchidism and HIR, following orchidopexy, with buserelin (a GnRH receptor agonist) normalized sperm parameters in 86% of the patients who otherwise would develop infertility, despite successful orchidopexy [Hadziselimovic, 2008]. Therefore, our results favor the hypothesis that *EGR4* is the master gene controlling fertility development. Although all 15 patients studied had isolated cryptorchidism (in comparably undescended position and identical age), the HIR group had a significantly lower *EGR4* expression indicating that an intact EGR4 function is not mandatory for the descent of the epididymo-testicular unit. This is a new observation that seriously questions the current dogma that the undescended position itself is the only factor responsible for infertility development.

In agreement with the decreased expression of *EGR4, DDX25/GRTH*, an RNA helicase involved in gene-specific mRNA export and protein translation during spermatogenesis was significantly downregulated in the HIR

group (table 2). *DDX25* is stimulated by LH/HCG via cyclic-AMP-induced androgen formation in testicular Leydig cells [Sheng et al., 2006]. The action of androgen on GRTH protein expression in germ cells appears specific to germ cells [Tsai-Morris et al., 2004].

CBL, CREM, DAZL, PIWIL2, RHOXF1, RNF17, TAF7L, TEX14 genes define distinct regulatory pathways of spermiogenesis, even though they may regulate some common target genes or transcripts. The function of each key regulator is testosterone dependent and affects the transcription or translation of multiple genes or transcripts important for the regulation of spermiogenesis. Furthermore, these genes were expressed at levels 5–8-fold lower in the HIR group than in the control group. Compared to the IIR group, the LIR group showed an increased transcription of *CYP26B1* which is known to be responsible for spermatogenesis development and, via retinoid signaling, it determines germ cell fate in mice [Bowles et al., 2006]. Interestingly, MAGE, GAGE, SSX and other cancer-testis associated proteins frequently expressed in many different types of cancer were shown to interact directly with the proliferation, differentiation and survival of human germ cells [Gjerstorff et al., 2008]. In the HIR group, consistent with impaired differentiation and development of spermatogonia the expression of all three CTA gene family members was down-regulated as well as the expression of the genes encoding follistatin, *ADA, ID4* and *NRG1* (table 2). Indeed, in the HIR group we observed that the expression of several genes involved in the GnRH regulation was absent or was downregulated: *FOXG1, ISL1, LHX6, NMU* and *WNT3* (table 2). Together with follistatin, activin establishes an intra-pituitary mechanism for controlling gonadotrophs [Winters and Moore, 2004]. However, FSH seems not to play as important a role in male fertility as does LH, since *FSHβ* knockout mice and FSH-receptor knockout mice are fertile, albeit with a reduced germ cell number and sperm quality [Kumar et al., 1997; Abel et al., 2000]. Nevertheless, at testicular level, two primary GnRH-regulated early response genes *JUN* and *ATF3*, as well as *CGA*, *LHB*, and *FSHB* from the tertiary network, were equally expressed in all groups.

In conclusion, mRNA expression analysis was used to identify genes involved in the pathogenesis of infertility in cryptorchidism. We identified differences in gene expression patterns between high and low infertility risk groups and reinforced the importance of EGR4 in the development of fertility. These findings further clarify the molecular pathophysiology of hypogonadotropic hypogonadism as the etiologic factor of infertility in cryptorchidism.

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