Original Paper

Urologia Internationalis

Urol Int 2010;84:353–361
DOI: 10.1159/000288242

Received: October 14, 2009
Accepted after revision: December 21, 2009

Decreased Expression of *FGFR1*, *SOS1*, *RAF1* Genes in Cryptorchidism

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Key Words
Cryptorchidism · *FGFR1* gene · *SOS1* gene · *RAF1* gene · Microarray

Abstract
Background: In recent years, several genes were found to be involved in the process of epididymo-testicular descent, the most frequently cited ones include *INSL3*, *HOXA10*, *GNRHR*, and *KAL1*. In this study, we analyzed the differences in gene expression profiles between cryptorchid and descended testes. In particular, we analyzed expression of all recently published genes known to be associated with undescended testis. ***Patients and Methods:*** Twenty-two testicular biopsies from 18 boys were analyzed. We analyzed gene expression in 16 cryptorchid and 6 descended testes using Affymetrix Human Genome U133 Plus 2.0 GeneChips, and validated the results with qPCR. ***Results:*** 3,688 transcripts were differentially expressed with an adjusted p value of <0.05 and a change of at least 1.5-fold. The list contained 1,866 downregulated and 1,822 upregulated transcripts in the cryptorchid testes. A novel observation in our study was that the fibroblast growth factor receptor 1 gene *(FGFR1)* and its mediators *SOS1* and *RAF1* were expressed less in undescended testes. ***Conclusion:*** Based on our results, it is possible that a subtle dysfunction (expression) of the *FGFR1*, *SOS1* and *RAF1* genes is involved in the development of the most common male reproductive tract disorder – unilateral or bilateral cryptorchidism.

Introduction

Cryptorchidism is the most frequent disorder of the urogenital tract in males. It is associated with important consequences in adulthood, such as infertility and testicular cancer. Androgens are key hormones involved in the completion of epididymo-testicular descent; therefore, impaired fetal androgen action can result in cryptorchidism. However, the underlying mechanism by which impaired androgen action produces 'isolated' cryptorchidism is still debatable [1]. Furthermore, fibroblast growth factor receptor 1 *(FGFR1)* gene mutations

N.O.H. and F.H. contributed equally to this work; N.O.H. and F.H. designed the research; N.O.H., P.D., E.J.O. and F.H. perfomed the research; Ch. de G., N.O.H. and P.D. contributed new reagents/analytic tools; N.O.H., P.D., E.J.O. and F.H. analyzed the data; F.H. wrote the paper.

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can cause both Kallmann syndrome and/or idiopathic hypogonadotropic hypogonadism, that are frequently associated with cryptorchidism [2]. Androgen-independent events may also be responsible for testicular descent. Insulin-like factor 3 (INSL3), and its receptor, relaxin family peptide receptor 2 (RXFP2, also called LGR8, leucine-rich repeat-containing GPCR, or GREAT, G-protein-coupled receptor), are possible regulators of testicular descent that may be responsible for isolated cryptorchidism [3]; although mutations in the *INSL3* and *RXFP2* genes have rarely been associated with human cryptorchidism. Overall, the frequency of *INSL3* and *RXFP2* mutations is 7/600 at birth (1.2%) and 7/303 (2.3%) in persistently cryptorchid males, with a higher prevalence in bilateral forms (5/120, 4.2%) than in unilateral forms (1/183, 0.5%) [3, 4]. In addition, cryptorchidism may be associated with a specific haplotype of the gene for estrogen receptor-α (ESR1), which mediates the estrogenic effects of environmental endocrine disrupters (EEDs). The effects of EEDs on testicular descent might depend on an individual's genetic susceptibility [5]. Epididymo-testicular descent is a process where both testis and epididymis descend from dorsal abdominal wall into the scrotum and in humans this process is accomplished at birth. 1–3% of epididymides and testes remain undescended and require treatment. Hormonal therapy generally around second birthday is successful in 20–60% depending on the undescended position of both organs. We hypothesize that undescended testis has different gene expression pattern compared to descended one. Therefore, we analyzed gene expression with Affymetrix method for all the genes known to be involved in epididymo-testicular descent.

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. 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. Cryptorchid testis was defined as a testis localized outside the scrotum and incapable of being brought into a stable scrotal position. Th e age of the patients at surgery ranged from 10 months to 4.5 years.

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 [6]. Not all contralateral testes in patients with testicular agenesis are expected to have normal histology and may require subsequent hormonal treatment [6]. Therefore, we routinely biopsy these testes during the period of testicular fixation performed in order to prevent testicular torsion.

Twenty-two testicular biopsies (as large as the size of a kernel of rice) from 20 boys were analyzed (16 testes from boys with cryptorchidism and 6 contralateral descended testes; 2 from boys with undescended testis and 4 from patients with testicular agenesis). All contralateral testes were selected for normal histology according to age. The mean age of the patients at surgery was 3.2 years and for the controls 3.8 years. All patients had an extensive clinical examination, and we could not find any clinical signs of developmental malformations or syndromes, none had hypospadias. In particular, no clinical 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 one abdominal, one inguinal and 14 at tubercle.

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 (Plan-Apochromat 63×/1.40 oil).

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 1× 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 [7]. Raw data files are available at the EBI ArrayExpress repository [8] via the accession numbers ETABM-130 and E-TABM-174 and GEO.

Statistical Analysis and Interpretation of Microarray Data

Data analysis and gene filtering were performed using R/Bioconductor [9]. Signal condensation was performed using only the RMA from the Bioconductor Affy package. Differentially expressed genes were indentified using the empirical Bayes method (Ftest) implemented in the LIMMA package and adjusted with the false discovery rate method [10]. 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 were performed in R.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed using a Corbett Research RG-6000 instrument. cDNAs were synthesized with Reverse Transcriptase – Core Kit (Eurogentec, cat.

Table 1. Antibodies utilized for immunohistochemical validation

Antibody	Animal	Code/lot	Type	Source
DDX25	goat	SC-51269/A2108	polyclonal	Santa-Cruz
EGR1	mouse	AB54966/509443	monoclonal	Abcam
EGR4	goat	SC-19868/JU306	polyclonal	Santa-Cruz
CBL	mouse	SC-1651/BO206	monoclonal	Santa-Cruz
ACVR1B1	rabbit	AB71539/603652	polyclonal	Abcam
ALDH1A2	mouse	SC-22591/1008	monoclonal	Santa-Cruz
KLF4	rabbit	AB26648/641290	polyclonal	Abcam
MAGEA4	goat	SC-28484/62006	polyclonal	Santa-Cruz
COL4A3	goat	SC-18177/L1404	polyclonal	Santa-Cruz
FGFR1	mouse	AB823	monoclonal	Abcam

No. RT-RTCK-03) using random primers. Real-time PCR runs were performed using SyBr Fast Kit (Kapa Biosystems, cat. No. 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 and 510 nm, respectively. Gain was set at 8.33. PCR program was set as follows: 95°C for 60 s – 45× (95°C for 3 s, 60°C for 10 s, 72°C for 4 s) followed by a melting curve analysis (65–95°C, rising by 0.65°C in 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.

Antibody Validation

We wanted to determine whether greater transcript expression corresponded to greater protein expression. Immunoperoxidase histochemistry, although not a quantitative method, is currently the only practical way to detect proteins in small fixed tissue samples. We employed ten antibodies to proteins known to be involved in the reproductive hormonal axis.

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 ten antibodies (table 1).

Secondary antibodies, labeled with polymer-horseradish peroxidase [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 on the basis of their nuclear morphology and position within the developing gonad. Immunohistochemistry experiments were performed at least twice on at least 4 patients from each group, and only those with identical results between experiments for each sample were considered acceptable. Controls for nonspecific 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 nonspecific 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 [11].

Ethical Considerations and Approval

In accordance with the Declaration of Helsinki, the Institutional Review Board of Kindertagesklinik 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

The total RNA and cRNA samples were of high quality, the signal intensity distributions were similar and were within the normal range. Figure 1 shows the heat map resulting from the visualized signal intensities, 3,688 transcripts were differentially expressed in the cryptorchid and descended testes after statistical filtering (fold change >1.5, adjusted p value <0.05). This gene list was composed of 1,822 elevated and 1,866 decreased transcripts in the cryptorchid testes.

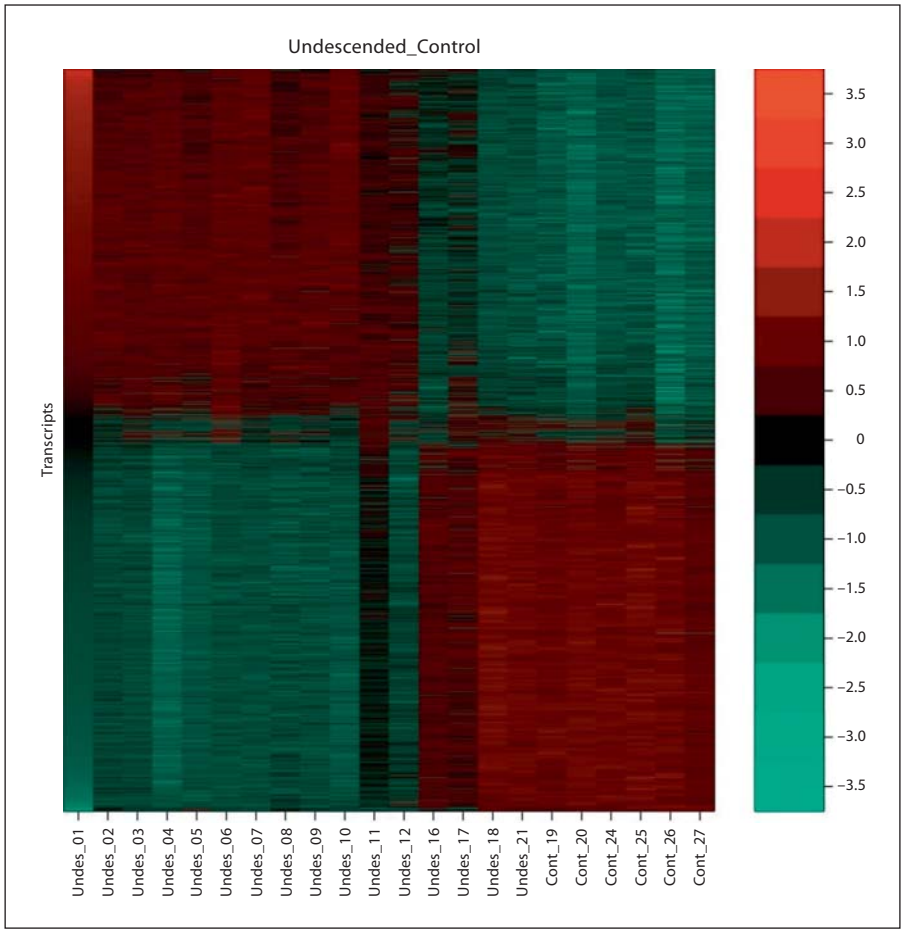

Fig. 1. Heat map showing gene expression levels of cryptorchid and descended testes. Red represents upregulated and green downregulated genes.

Our particular interest was to investigate the expression profiles of genes that had previously been shown to be associated with cryptorchidism, namely: *INSL3, RXFP2/LGR8/GREAT, NR4A1, NR5A1, CYP19, NROB1/DAX1/AHC, KAL1, GNRHR, PROK2, LHB, ESR1, SOX2, GPR54,* and *HOXA10* (table 2). We found *INSL3* was expressed equally in descended and undescended testes. Furthermore, analysis of the expression pattern of the gene for the INSL3 receptor, *RXFP2*, also called *LGR8* or *GREAT*, showed no differences between the two groups studied (table 2). Low, not significantly different expression was found in both groups for *CYP19A1*, *GNRHR*, *PROK2*, *SOX2, KISS1R* (*GPR54*), and *HOXA10* (table 2). No differences were observed in the signaling intensity of *NR4A1*, *NR5A1*, *NROB1/DAX1/AHC*, *KAL1*, *LHB*, and *ESR1* (table 2). Our main finding was that cryptorchid testis displayed reduced expression of *FGFR1*, as well as *RAF1* and *SOS1*, known to be part of its signaling pathway (table 3).

Immunohistochemical and qRT-PCR Validation

Immunoperoxidase histochemistry, although not a quantitative method, is currently the only practical way to detect proteins in small fixed tissue samples. To determine whether transcript expression corresponded to protein expression, several antibodies were employed and the histological sections of both groups were compared. The proteins EGR4, MAGE4, ALDH1, and COL4A3 were expressed in the spermatogonia of both groups (fig. 2). Identical cytoplasmic localization was found for all other proteins analyzed. In addition to its expression in germ and Leydig cells, CBL was expressed in Sertoli cells. Fgfr1 expression was observed in cytoplasm of the germ and Leydig cells (fig. 3). We validated our microarray data by qRT-PCR on four genes that showed significant expression changes, all of which showed high correlation in the obtained transcriptional profiles (fig. 4).

Discussion

The hypothalamo-pituitary-gonadal axis (HPG) regulates the development and the endocrine and reproductive functions of the gonads throughout all phases of life. Several animal models of hypogonadotropic hypogonadism are associated with cryptorchidism. (1) HPG male mice lacking *GnRH* are cryptorchid, but have a normal gubernaculum, and their testes develop and descend normally if treated with gonadotropins [12]. (2) Pask et al. [13] reported a *GnRH receptor (GnRHr)* gene loss-of-function mutation in male mice that caused hypogonadotropic hypogonadism during *N*-ethyl-*N*-nitrosourea mutagenesis screening. Affected males had a micropenis, undescended testes, and were infertile. (3) Luteinizing hormone receptor *(LHR)* knockout male mice showed undescended testes, small testis, underdeveloped scrotum, small penis, and arrested spermatogenesis [14, 15]. Cryptorchidism in *LHR* knockouts was caused by developmental defects of gubernaculum because of testosterone deficiency, and testosterone replacement therapy reversed all morphologic and gene expression changes except *INSL3*, suggesting that testosterone secreted by Leydig cells facilitates the completion of testicular descent [15]. These knockout male mice had inguinoscrotal testes, suggesting the HPG axis is distributed in the inguinoscrotal phase of testis descent [15].

The FGFR1 signaling pathway regulates cell proliferation, migration, differentiation, and survival; accordingly, it is essential for various phases of human development [16]. Mutations of the *FGFR1* gene have been described in cases of idiopathic hypogonadotropic hypogonadism with functioning olfactory bulb and normal sense of smell [2, 17]. Pitteloud et al. [17] described that FGFR1 was critical not only for the migration of GnRH neurons across the olfactory tracts, but also demonstrated that an entirely different mechanism must exist for the failure of GnRH in those cases with normal olfactory bulbs, tracks, and senses of smell [18]. This phenotypic complexity suggested *FGFR1* mutations, in addition to presumably playing a critical role in GnRH neuronal maturation, may be associated with neuronal survival and possibly apoptosis in normosmic idiopathic hypogonadotropic hypogonadism patients with *FGFR1* mutations [19].

Leydig cell atrophy was observed in all undescended testes (fig. 3, 4). This atrophy is a consequence of hypogonadotropic hypogonadism [20, 21]. Furthermore, low number of germ cells and particularly impaired germ cell differentiation is a further evidence of impaired gonadal maturation after birth, also denominated mini-puberty (fig. 3, 4) [20, 21].

Table 2. Expression values of 16 genes involved in the process of epididymo-testicular descent

Gene symbol	Probe set ID	Median value	
		cryptorchid	descended
INSL3	1553594_a_at	9.55051762	9.44872353
	214400_at	8.96849613	8.59509113
	214572_s_at	10.2362787	9.98199254
RXFP2	1553326_at	2.51231563	2.49421155
NR4A1	202340_x_at	6.17169726	6.35677944
	210226_at	5.44048775	5.47187537
	211143_x_at	4.95417354	4.73797538
NR5A1	210333_at	5.01168715	4.50725008
CYP19A1	1554296_at	2.21019698	2.18203546
	1560295_at	4.01860367	4.17272721
	203475_at	2.86980186	2.8237
	240705_at	3.50708023	3.40659826
	240863_at	2.17002677	2.15669923
NR0B1/DAX1/AHC	206644_at	4.10496977	3.50749603
	206645_s_at	7.52022053	6.39801481
KAL1	205206_at	9.49971782	9.25717797
	229853_at	3.59942868	3.62896527
GNRHR	211522_s_at	3.75227569	3.08793908
	211523_at	2.70213402	2.64301747
	216341_s_at	4.65314009	4.57382752
PROK2	232629_at	3.89249056	3.86664277
LHB	214471_x_at	5.5269635	5.29761942
ESR1	205225_at	5.69431462	5.20809283
	211233_x_at	4.49075955	4.24512071
	211234_x_at	5.07846696	5.04469176
	211235_s_at	4.9183534	4.86495925
	211627_x_at	2.83001718	2.64012202
	215551_at	2.99613765	3.22418077
	215552_s_at	3.30187431	3.28320076
	217163_at	3.69396359	3.57172417
	217190_x_at	3.38580973	3.07410043
SOX2	213721_at	3.81689992	3.66312997
	213722_at	3.50821965	3.45568233
	214178_s_at	3.88332337	4.02062008
	228038_at	2.59151923	2.5668031
KISS1R (GPR54)	242517_at	3.27949914	2.94987107
HOXA10	213147_at	4.57090229	4.48878348
	213150_at	3.63283876	3.14758548

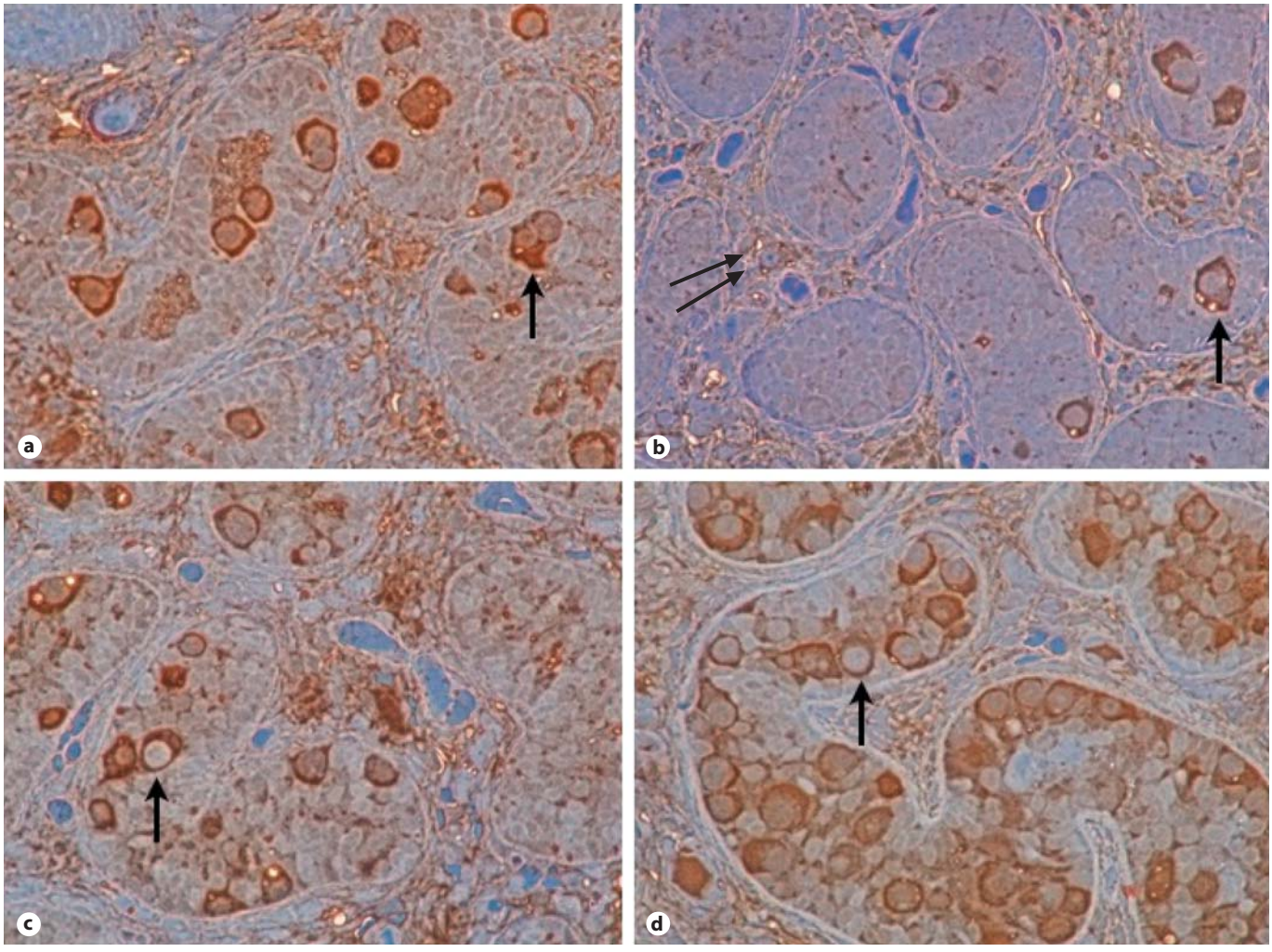

Fig. 2. Gene validation at protein level. **a** MAGE4. **b** EGR4. **c** ALDH1A2. **d** COL4A3. All four antibodies were found in the cytoplasm of germ cells stained brown (arrow). Contra-staining was performed with toluidine blue. No EGR4 in Sertoli cells or peritubular connective tissue. Atrophic juvenile Leydig cell (double arrow).

Table 3. FGFBP2, FGFR1, RAF1, SOS1 gene expression values of cryptorchid and descended testes

Gene symbol	Probe set ID	Median value		Adjusted p value	logFC
		cryptorchid	descended		
FGFBP2	223836_at	7.61253	6.25911	0.04170411	1.52937555
FGFR1	215404_x_at	6.06631	7.17697	0.00848763	–1.02287333
FGFR1	222164_at	5.54525	6.31121	0.01670167	–0.72301012
RAF1	1557675_at	6.72658	7.61658	0.0399879	–0.79031992
SOS1	230337_at	7.20646	8.32818	0.03287241	–0.92108813

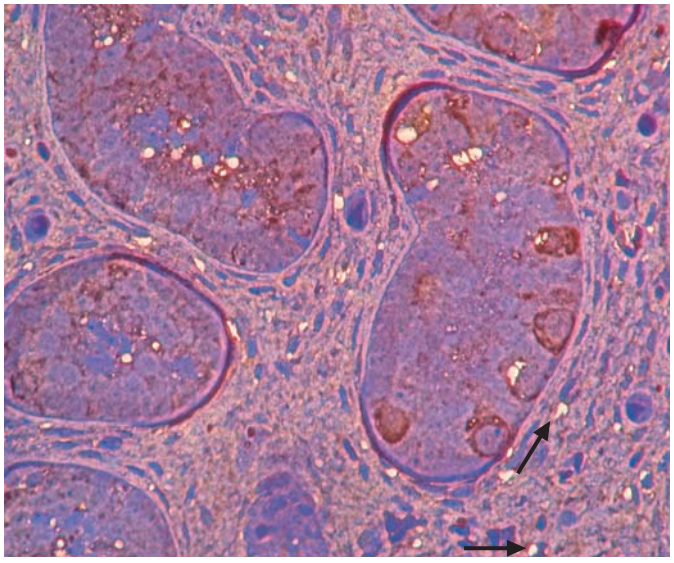

Fig. 3. FGFR1 antibody found in the cytoplasm of germ cells stained brown, as well as in atrophic Leydig cells (arrows) and peritubular connective tissue.

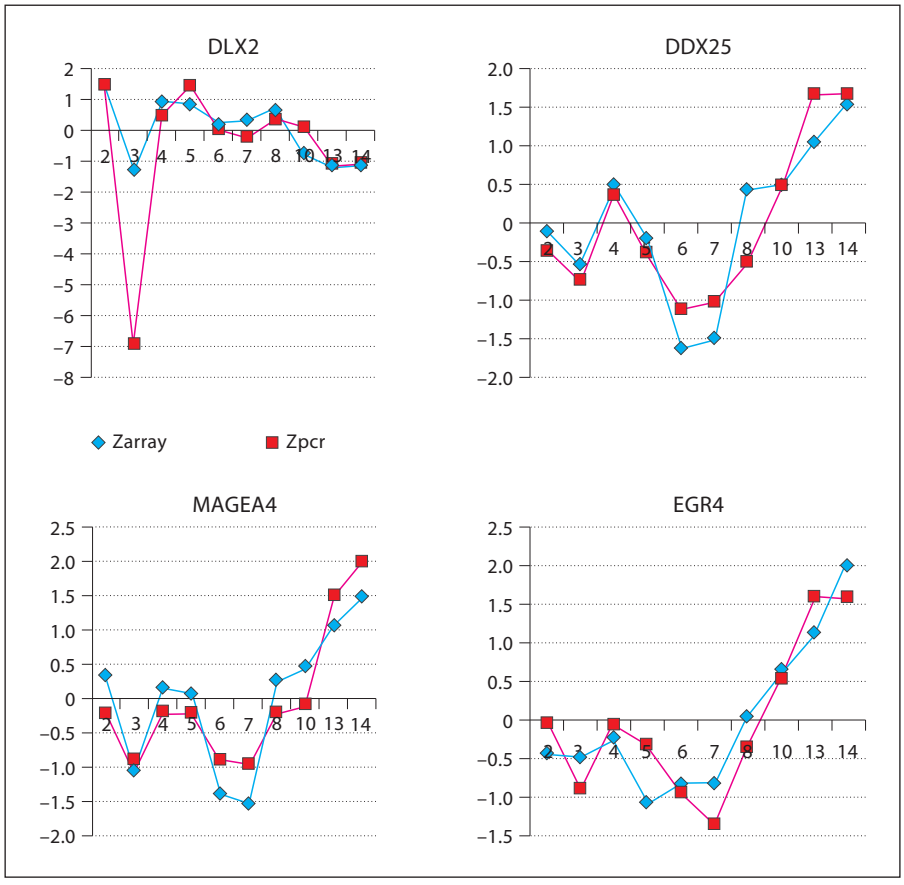

Fig. 4. Correlation of microarray data with data obtained by qRT-PCR on four genes that displayed significant expression cha nges showing a high correlation in the obtained transcriptional profiles. The y-axis is z-normalized Log2 expression (array) and 2-normalized QT value (QPCR); x-axis shows biopsy file names.

Furthermore, FGFR1 affects the proliferation and migration of vascular smooth muscle cells [22] and is involved in myoblast proliferation and differentiation [23]. Inhibited muscle development was observed in the epididymis and scrotal anlage in *INSL3* mutant mice [24–26]. Reduced expression of *FGFR1* has not been described before in association with isolated cryptorchidism. Nevertheless, mediators of the FGFR1 signaling pathway, SOS1 and RAF1, have been associated with leopard syndrome and Noonan syndrome [27], syndromes that include cryptorchidism. Theoretically, the reduced gene expression of *FGFR1* and its mediators, *SOS1* and *RAF1*, could affect muscle development and, therefore, cause crypto-epididymis.

Recently, Ferlin et al. [3] determined the frequency of genetic alterations such as karyotype anomalies and *INSL3*, *LGR8/GREAT*, and *AR* gene mutations in cryptorchidism in their large study. They reported that the overall frequency of genetic alterations was significantly higher in boys with cryptorchidism (5.3%) than in controls (0.3%). As a result, the odds ratio for the association of cryptorchidism with genetic alterations was 16.7, indicating a significant association between cryptorchidism and genetic alterations. In more than 2,000 patients and controls analyzed to date, the T222P *RXFP2* mutation is the only mutation strongly associated with the mutant phenotype. INSL3 production is also related to LH [2]; although INSL3 apparently has a great effect in mouse models, it remains unclear what role it plays in the testicular descent of humans. Analyses of mutations of *INSL3*, as well as *LGR8/GREAT* genes, in boys with either unilateral or bilateral cryptorchidism revealed only a small percentage to have sequence changes of functional significance. Our examinations showed no differences in the gene expression profiles of *INSL3* or *LGR8* between subjects with descended and cryptorchid testes. In all of the boys we examined, the transabdominal phase had been completed. This could explain why we could not find a difference in expression of *INSL3*.

We could not find significant differences between descended and undescended testis with respect to the 14 genes described as being involved in cryptorchidism development. This may be because these genes were found associated either with pronounced androgen deficiency *(NR5A1*, *NR4A1*, and *CYP19)* or with classical hypogonadotropic hypogonadism *(GNRHR*, *KAL1*, *LHB*, and *PROK2)*.

In conclusion, mutations of the *FGFR1* gene induce idiopathic hypogonadotropic hypogonadism. A subtle dysfunction of the *FGFR1* gene, observed here in cryptorchid testes, may be responsible for unilateral or bilateral epididymo-testicular arrest along the descent path into the scrotum.

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