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ORIGINAL ARTICLE

Comparison of global gene expression profiles of microdissected human foetal Leydig cells with their normal and hyperplastic adult equivalents

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STUDY QUESTION: Do human adult Leydig cells (ALCs) within hyperplastic micronodules display characteristics of foetal LCs (FLCs)?

SUMMARY ANSWER: The gene expression profiles of FLCs and all ALC subgroups were clearly different, but there were no significant differences in expressed genes between the normally clustered and hyperplastic ALCs.

WHAT IS KNOWN ALREADY: LCs are the primary androgen producing cells in males throughout development and appear in chronologically distinct populations; FLCs, neonatal LCs and ALCs. ALCs are responsible for progression through puberty and for maintenance of reproductive functions in adulthood. In patients with reproductive problems, such as infertility or testicular cancer, and especially in men with high gonadotrophin levels, LC function is often impaired, and LCs may cluster abnormally into hyperplastic micronodules (defined as clusters of >15 LCs in a cross-section).

STUDY DESIGN, SIZE, DURATION: A genome-wide microarray study of LCs microdissected from human foetal and adult tissue samples ($n = 12$). Additional tissue specimens ($n = 15$) were used for validation of the mRNA expression data at the protein level.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Frozen human tissue samples were used for the microarray study, including morphologically normal foetal (gestational week 10–11) testis samples, and adult testis specimens with normal LC distribution, LC micronodules or LC micronodules adjacent to hCG-producing testicular germ cell tumours. Transcriptome profiling was performed on Agilent whole human genome microarray 4 × 44 K chips. Microarray data pre-processing and statistical analysis were performed using the limma R/Bioconductor package in the R software, and differentially expressed genes were further analysed for gene set enrichment using the DAVID Bioinformatics software. Selected genes were studied at the protein level by immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: The transcriptomes of FLCs and ALCs differed significantly from each other, whereas the profiles of the normally clustered and hyperplastic ALCs were similar despite morphological heterogeneity. The study revealed several genes not known previously to be expressed in LCs during early development, including sulfotransferase family 2A member 1 (*SULT2A1*), WNT1-inducible signalling pathway protein 2 (*WISP2*), hydroxyprostaglandin dehydrogenase (*HPGD*) and insulin-like growth factor 2 mRNA binding protein 1 (*IGF2BP1*), whose expression changes were validated at the protein level.

LARGE SCALE DATA: The transcriptomic data are deposited in ArrayExpress (accession code E-MTAB-5453).

[†]These authors contributed equally to this study.

LIMITATIONS, REASONS FOR CAUTION: The small number of biological replicates and the necessity of RNA amplification due to the scarcity of human tissues, especially foetal specimens, are the main limitations of the study. Heterogeneous subpopulations of LCs within micronodules were not discriminated during microdissection and might have affected the expression profiling. The study was constrained by the lack of availability of truly normal controls. Testis samples used as 'controls' displayed complete spermatogenesis and were from patients with germ cell neoplasia but with undetectable hCG and normal hormone levels.

WIDER IMPLICATIONS OF THE FINDINGS: The changes in LC morphology and function observed in patients with reproductive disorders possibly reflect subtle changes in the expression of many genes rather than regulatory changes of single genes or pathways. The study provides new insights into the development and maturation of human LCs by the identification of a number of potential functional markers for FLC and ALC.

STUDY FUNDING AND COMPETING INTEREST(S): The study was supported by research grants from the Danish Cancer Society, the Capital Region's Research Fund for Health Research, Rigshospitalet's research funds, the Villum Kann Rasmussen Foundation, the Danish Innovation Fund, ReproUnion, Kirsten and Freddy Johansen's foundation and the Novo Nordisk Foundation. None of the funding agencies had any influence on the study. The authors declare no conflicts of interest.

Key words: Leydig cell / human testis / gene expression profiling / transcriptome / foetal Leydig cell / Leydig cell micronodules / Leydig cell hyperplasia / testicular germ cell tumours

Introduction

Leydig cells (LCs) in the testis secrete hormones and paracrine factors, including androgens, which are essential for normal male development and reproductive functions. In humans, the LCs appear in three separate waves of proliferation and differentiation, and the populations are distinguished as foetal LCs (FLCs), neonatal LCs (in primates only) and adult LCs (ALCs). Each of the waves corresponds to a peak in circulating testosterone levels during intrauterine development, mini-puberty and young adulthood, respectively (Prince, 2001). It is still a matter of debate whether the different human LC populations not only are separated in time but also derived from different cell lineages (Griswold and Behringer, 2009; Teerds and Huhtaniemi, 2015). Differences in expression of selected genes (Tapanainen *et al.*, 1989), as well as steroid metabolism pathways (Tapanainen *et al.*, 1981), have been observed between human FLCs and ALCs. But in contrast to several detailed gene expression studies of FLCs in rodents (Jameson *et al.*, 2012; McDowell *et al.*, 2012; Sanz *et al.*, 2013; McClelland *et al.*, 2015; Inoue *et al.*, 2016), only one published study investigated the global transcriptome of human foetal testes and ovaries (Houmard *et al.*, 2009). To our knowledge, no study compared global gene expression profiles of human FLCs and ALCs.

Decreased LC function is common in men with reproductive disorders, including testicular dysgenesis syndrome (TDS), which comprises those cases of subfertility, cryptorchidism, hypospadias and testicular cancer that are pathogenetically linked to impaired testis development (Skakkebaek *et al.*, 2001; Joensen *et al.*, 2008). Impaired LC function is manifested as a decreased testosterone/LH ratio and the presence of LC micronodules in the testis (Holm *et al.*, 2003). The number and size of LC micronodules, defined as clusters of >15 LCs in a cross-section, increases with the severity of the TDS and with increasing gonadotrophin levels (Holm *et al.*, 2003; Lardone *et al.*, 2013). In some patients with testicular germ cell tumours (TGCTs), the formation of LC micronodules can be further stimulated by the high secretion of hCG by a subset of these tumours, especially nonseminomas with a choriocarcinoma component. Regardless of the tumour type and hCG levels, LC micronodules are observed in the contralateral testis in 75% of patients with TGCT (Hoei-Hansen *et al.*, 2003). This emphasizes the high frequency of LC micronodules and suggests possible influence of impaired LC development and function that precedes the TGCT formation.

Morphological changes have been demonstrated in LCs within micronodules suggesting failure of final maturation. These changes include decreased smooth endoplasmatic reticulum, an irregularly indented nuclear membrane, and decreased lipofuscin pigment granules and Reinke crystals (Kozina *et al.*, 2011; Soerensen *et al.*, 2016). In addition, the proportion of morphologically abnormal LCs was inversely correlated with testosterone levels in patients with primary testicular disorders often associated with LC micronodules, including cryptorchidism, Klinefelter syndrome and Del Castillo's syndrome (Paniagua *et al.*, 1984).

A few molecular factors, including transforming growth factor beta 1 (TGFB1) signalling, have been implicated in the formation of LC hyperplasia (Gonzalez *et al.*, 2010a,b), but the mechanisms are still unknown. A comprehensive study of gene expression changes in the human hyperplastic or hypertrophic LCs has never been performed.

Our previous studies of TGCT and in particular their precursor, germ cell neoplasia *in situ* (GCNIS), previously known as carcinoma *in situ* (CIS), suggested a strong association of this neoplasia with impaired testis development (Skakkebaek *et al.*, 1987; Rajpert-De Meyts, 2006). However, we do not know whether morphological changes of LCs within micronodules associated with TGCTs reflect a developmental disorder, or if they arise secondary to GCNIS or TGCT formation. Immature ALCs and FLCs have morphological similarities such as a lipid rich cytoplasm (Haider, 2004) and both express proteins not detectable in the mature ALCs (Lottrup *et al.*, 2014). We hypothesized that at least a subset of LCs within micronodules have an immature phenotype, or might even be remnants of FLC, as postulated in mice (Shima *et al.*, 2015; Wen *et al.*, 2016). This hypothesis is based on the presence of other immature cell types in testis tissue of patients with TDS, such as GCNIS cells, which are foetal gonocyte-like arrested germ cells (Sonne *et al.*, 2009a) or Sertoli cells, which often are undifferentiated in patients with TGCT (Hoei-Hansen *et al.*, 2003; Tarulli *et al.*, 2013). To address this hypothesis, we performed global gene expression profiling of human FLCs and three different ALC populations: normally sized LC clusters, and micronodules found in the vicinity of hCG-producing or hCG-negative TGCTs. Because of the lack of access to suitable frozen material from patients with truly normal LCs, e.g. in obstructive azoospermia, all ALC samples (except one) were obtained from patients with germ cell neoplasia, either

in situ or with overt TGCT. However, care was taken to select as 'controls' tissue specimens from patients with complete spermatogenesis, undetectable hCG and normal hormone levels. In the data analysis, we identified genes that were differentially expressed between the study groups and revealed possible functional differences. Further, selected differentially expressed candidate genes were validated at protein level in an independent set of tissue specimens. We present here the data and analysis of the results.

Materials and Methods

Tissue samples and ethical considerations

Adult samples were residual tissues from orchidectomy specimens collected at the Department of Pathology at Rigshospitalet from patients diagnosed with a TGCT or pre-invasive GCNIS, after their written informed consent. After evaluation by pathologists, the remaining tissue fragments were snap-frozen or fixed and paraffin-embedded, and stored at the Department of Growth and Reproduction, Rigshospitalet. Foetal gonads were kindly provided by Dr Ludmila Ruban and Professor Harry D. Moore, University of Sheffield, UK, in connection with a previous collaborative study (Sonne *et al.*, 2009a). Collection of human foetal gonads in the UK was carried out in agreement with the Polkinhorne guidelines and following ethical approval and written informed consent. The foetal tissues were obtained from social abortions, and the gestational age was estimated based on the first day of the last period.

A total of 12 frozen human testicular samples were examined by microarrays; 3 adult specimens with normally clustered LCs (denoted 'normal ALC'), 3 adult specimens with LC micronodules ('LC micronodules'), 3 adult specimens with LC micronodules adjacent to an hCG-producing tumour ('LC/TGCT+hCG') and 3 frozen foetal gonads from gestational week (GW) 11–13 ('FLC') (Table I).

For validation of the results by immunohistochemistry (IHC), a separate set of 10 adult and 5 foetal paraffin-embedded testis tissue samples from the biobank of Departments of Growth and Reproduction or Pathology were used. The foetal gonads were obtained from social abortions at GW 10–11, or at autopsy of miscarriages (GW 15–25) for reasons unrelated to the foetal development. Following surgical excision, the samples were immediately fixed in formalin, dehydrated and embedded in paraffin.

The study was approved by the regional medical and research ethics committee (permit no. H-2-2009-137) and the Danish Data Protection Agency (no. 2012-41-1390).

Microdissection of tissues and assessment of cellularity

Excised adult testicular tissue samples and foetal gonads were embedded in OCT compound (Sakura Fintek Europe, Zoeterwonde, The Netherlands) and snap-frozen at −80°C in isopentane. Prior to laser capture microdissection (LCM), sections with thickness of 6–10 µm (foetal tissue) and 16–18 µm (adult tissue) were cut using a Microm HM550 Cryotome (Thermo Scientific, Kalamazoo, MI, USA) onto nuclease- and nucleic acid-free membrane slides (Molecular Machines & Industries, Zurich, Switzerland). The sections were immediately fixed in 75% ethanol (EtOH) for 10 min and stored in 100% EtOH at −80°C. All procedures were performed using baked glassware and water treated with diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, MO, USA). Serial sections of each tissue were collected on 'super frost' glass before and after the collection on membranes and stored at −20°C for subsequent histological assessment of cellularity. These slides were used as a reference to visualize the abundance of LCs in the different samples, and to validate the staining method used for the microdissected samples (see below).

Adult and foetal samples were treated slightly differently prior to microdissection. Adult testis sections were transferred to room temperature

Table I Microdissected samples of human foetal LCs and their normal and hyperplastic adult equivalents for microarray analysis.

Samples	Age	*LC micronodules	Serum hCG (IU/l)	Diagnosis
FLC	GW 11–12	–	–	–
FLC	GW 11–12	–	–	–
FLC	GW 12–13	–	–	–
Normal ALC	42 y	− to +	<2	SEM
Normal ALC	25 y	–	<2	GCNIS
Normal ALC	24 y	− to +	<2	GCNIS
LC micronodules	43 y	+++++	<2	LC hyperplasia****
LC micronodules	17 y	+++	<2	GCNIS
LC micronodules	38 y	+	<2	SEM
LC/TGCT + hCG**	27 y	+++++	N/A***	SEM
LC/TGCT + hCG	39 y	++	41	SEM
LC/TGCT + hCG	28 y	+++	8	SEM

*The clustering and number of Leydig cells (LCs) was assessed as no micronodules (−), no true micronodules but subtle increase in LC numbers (−/+), few micronodules (+), increasing numbers of micronodules with barely no tubular structures left in the most severe samples (++ to +++++).
**Supraphysiologically stimulated LC micronodules from testis with an hCG-producing tumour.
***Serum hCG was not analysed in this patient, however, a hormone profile with undetectable gonadotrophin levels was consistent with the presence of an hCG-producing tumour.
****The patient was incorrectly diagnosed with TGCTs, the following evaluation revealed no germ cell malignancy, but extensive LC hyperplasia.
FLC, foetal LC; ALC, adult LC; GW, gestational week; y, years; TGCT, testicular germ cell tumour; SEM, seminoma; GCNIS, germ cell neoplasia *in situ*.

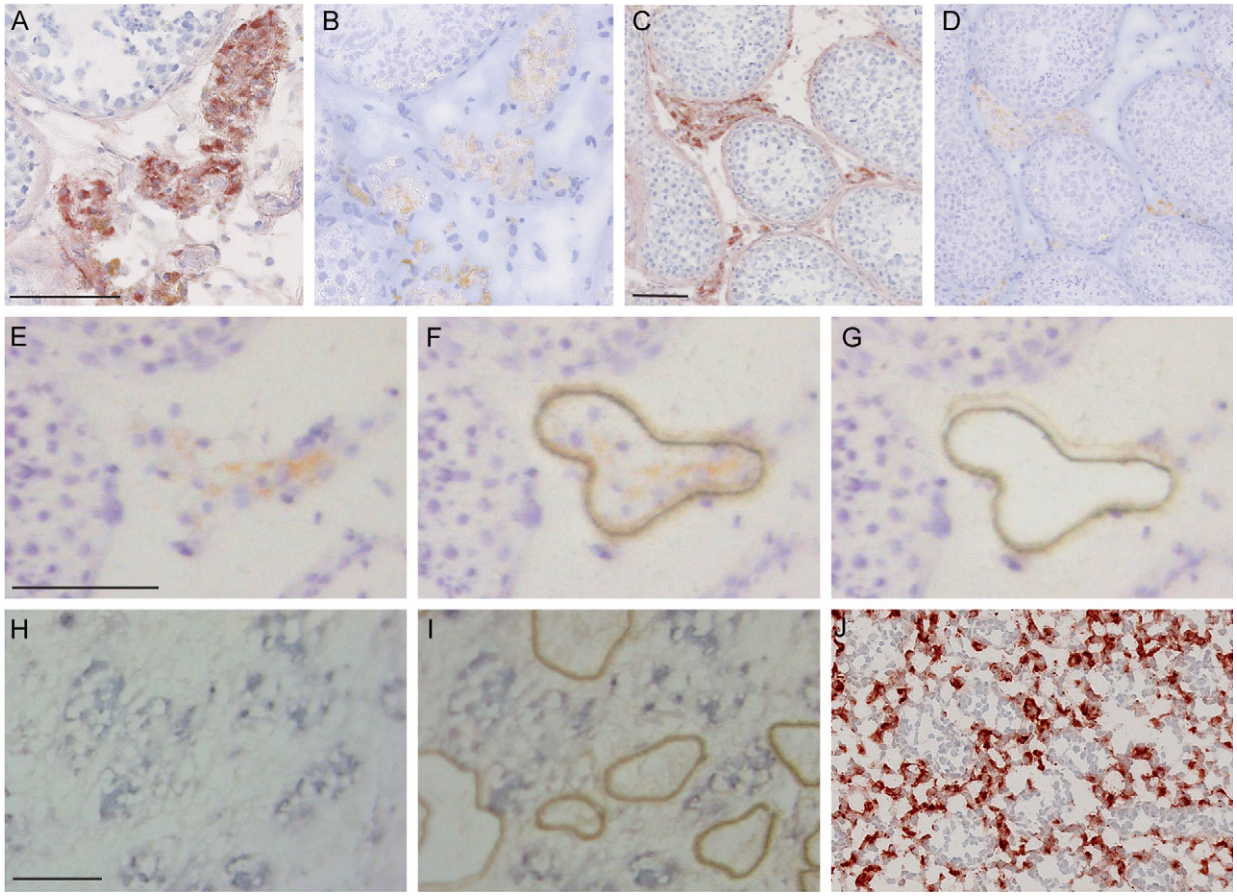

Figure 1 Isolation of human Leydig cells (LCs) by laser microdissection. Images (**A**–**D**) show serial sections of frozen adult testis tissue in a high (A, B) or low (C,D) magnification; reference slides were stained by immunohistochemistry (IHC) for cytochrome P450 family 11 subfamily A member 1 (CYP11A1) to visualize the LCs (red in A, C), or with haematoxylin only (B, D). The latter staining protocol was used for samples that were dissected taking advantage of a brown-red natural colour of the adult LCs. Images (**E**–**G**) (middle row) show a frozen adult testis placed on a membrane slide, before (E), during (F) and after (G) dissection by laser microdissection (LCM). The bottom panel (**H**, **I**) illustrates the LCM procedure in a frozen foetal testis specimen; the sections placed on membrane slides were stained with NBT-BCIP to visualize gonocytes (blue in H, I), and the spaces devoid of colour were microdissected (I). Image (**J**) shows a reference serial section stained for CYP11A1 by IHC to visualize the LCs (red). Scale bars in images A, C, E and H represent 100 μm. Image B has the same magnification as A, image D has the same magnification as C, images F and G have the same magnification as E, and images I and J have the same magnification as H.

and stained with Meyer's haematoxylin and dehydrated as follows: 95% EtOH 10 s, 75% EtOH 10 s, DEPC-treated H_2O 10 s, Meyer's haematoxylin 5 s, DEPC treated H_2O 10 s, 75% EtOH 10 s, 95% EtOH 10 s, 100% EtOH 10 s and air dried at room temperature. Foetal tissue sections were transferred to room temperature and stained with nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate mixture (NBT-BCIP) to visualize alkaline phosphatase-positive gonocytes, following previously published protocol (Sonne *et al.*, 2009b). This step required pretreatment in revelation buffer 10 s, followed by NBT-BCIP solution 90–120 s, DEPC H_2O 10 s, 62% EtOH 10 s, 96% EtOH 2 × 10 s, 100% EtOH 2 × 10 s and air drying.

To estimate cellularity of the specimens used for microdissection, the reference slides were prior to dissection carefully examined regarding tissue morphology and the expression of markers for different cell types (see IHC staining protocol below). The LCs were dissected from tissue sections at room temperature using the LMD CellCut and SmartCut systems (Olympus/Molecular Machines & Industries). For each specimen, one to three whole sections were cut directly from the membrane, but otherwise treated like the LCM samples and used to assess RNA integrity of the tissue specimens, as the RNA integrity of the microdissected samples is difficult to assess due to the low RNA concentration.

During microdissection, the LCs in the adult tissue were identified based on their location, morphology and their natural brown-red colour (Fig. 1A–D). The brown-red colour is probably due to a high content of lipofuscin or accumulation of lipid droplets (Miquel *et al.*, 1978) but should not be mistaken with the orange colour of erythrocytes that may be observed in traumatized and bloody samples. The brown-red colour of the LCs is retained during fixation in ethanol, which makes it possible to identify LCs in sections only stained with an aqueous solution of Meyer's haematoxylin (Fig. 1E–G). The LCs in the foetal samples did not naturally have any colour; hence these sections were stained by NBT-BCIP, which marks the alkaline phosphatase-positive foetal germ cells (gonocytes). The FLCs were identified based on morphology and location outside cords with

gonocytes, within NBT-BCIP-negative compartments of the stained tissue sections, which in the serial reference slides were positive for an LC marker, cytochrome P450 family 11 subfamily A member 1 (CYP11A1) (Fig. 1H–J).

The LC content of the microdissected tissue samples was assessed by evaluation of the distribution of cell populations on the reference slides, stained using IHC for CYP11A1, delta like non-canonical Notch ligand 1/foetal antigen 1 (DLK1/FA1), cluster of differentiation 99 (CD99, MIC-2), and by visual slide inspection during LCM. The unavoidable contamination of the LC pool by other cell types in the interstitium, including mesenchymal cells, epithelial cells from small vessels, macrophages and lymphocytes, was assessed by morphological assessment of the fixed reference slides. The purity of LCs dissected from the adult testis was estimated as very good in the normal samples but likely less so in LC micronodules, because of the greater content of small vessels in the latter. The purity of the dissected FLC samples was estimated to be lower than that of the ALC samples due to the abundance of mesenchymal cells in the interstitial compartment at this point in development.

After microdissection, the collected cells were immediately dissolved in 50–100 µl lysis solution from the RNAqueous micro kit (Life Technologies/Ambion, Carlsbad, CA, USA) and stored at −80°C.

IHC

The serial sections prepared as reference slides to establish the histology and cellularity of the frozen specimens used for microdissection were stained by IHC using a standard indirect peroxidase method. Briefly, all sections were thawed at room temperature for 5 min and fixed in formalin. Afterwards, all sections were incubated with 0.5% H_2O_2 (VWR, Fontenay-sous-Bois, France) to inhibit endogenous peroxidase activity, followed by incubation with 2% non-immune goat serum (Zymed Histostain kit, San Francisco, CA, USA) to minimize cross-reactivity, and incubated overnight with the primary antibodies. The following antibodies were used as markers for different cell types: rabbit-anti-human CYP11A1 1:500 (HPA016436, Atlas Antibodies AB, Sweden) for all LCs, rabbit-anti-human DLK1/FA1 1:2000 (provided by Charlotte Harken Jensen, University of Southern Denmark, Denmark) for foetal and immature LC (Jensen *et al.*, 1999; Lottrup *et al.*, 2014) and mouse-anti-human MIC-2 1:400 (M3601, DAKO, Denmark) diluted in Tris-buffered saline (TBS) for Sertoli cells (Visfeldt *et al.*, 1999). The following day, sections were incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Zymed Histostain kit) before a peroxidase-conjugated streptavidin complex (Zymed Histostain kit) was used as a tertiary layer. Visualization was performed with amino ethyl carbasole (Zymed Histostain kit). The sections were washed in TBS (3 × 2 min) between each step. All slides were counterstained with Meyer's haematoxylin.

A similar IHC procedure was used for validation of the array data at the protein level in archival paraffin-embedded tissue blocks, except that thinner (4 µm) sections were cut, deparaffinized and rehydrated, and an additional step of antigen retrieval was performed by microwaving the sections for 15 min before the 0.5% H_2O_2 incubation step. The following primary antibodies were used: rabbit-anti-human sulfotransferase family 2A member 1 (SULT2A1) 1:200 (HPA041487, Atlas Antibodies AB, Sweden), rabbit-anti-human hydroxyprostaglandin dehydrogenase (HPGD) 1:200 (HPA005679, Atlas Antibodies AB, Sweden), mouse-anti-human insulin-like growth factor mRNA binding protein 2 (IGF2BP1/IMP-1) 1:50 (sc-166344, Santa Cruz Technology, CA, USA) and rabbit-anti-human WNT1-inducible signalling pathway protein 2 (WISP2) 1:50–1:200 (sc-25442, Santa Cruz Technology, CA, USA).

Gene expression microarrays

RNA was purified using the Ambion RNAqueous micro kit (Life Technologies/Ambion, Carlsbad, CA, USA) and RNA quality was analysed by the Bioanalyzer Pico-kit (Agilent Technologies, Santa Clara, CA, USA). The samples were amplified in two rounds using the (Amino Allyl) MessageAmp™ II aRNA Amplification Kit (Life Technologies/Ambion, Carlsbad, CA, USA) with 14 h of *in vitro* transcription. The second round of amplification was performed with aminoallyl labelled UTP in the nucleotide mix, which was labelled with Cy3 (Amersham Biosciences, Uppsala, Sweden) as described in the protocol for Amino Allyl MessageAmp™ II aRNA Amplification kit. The labelled samples were applied to Agilent whole human genome microarray 4 × 44 K chips for transcriptome profiling (Design Number 014850, Agilent Technologies). Hybridization and scanning were performed as described by the manufacturer (Agilent Technologies) and analysed using the Agilent Feature extraction software (Agilent Technologies).

Gene expression data analysis

The microarray data analysis was performed using the freely available R software suite version 2.14.1 (http://www.r-project.org). Briefly, the gProcessedSignals were loaded into the limma R/Bioconductor package (Bolstad *et al.*, 2003; Gentleman *et al.*, 2004; Ritchie *et al.*, 2015). The data were normalized between arrays using the quantile normalization method (Smyth and Speed, 2003; Smyth, 2004) and the probes were collapsed for each systematic transcript ID by taking the median expression value. Principal component analyses (PCA) were performed using the R *prcomp* function with standard parameters. Hierarchical clustering (HCL) was performed on calculated Euclidian distances using the R *hclust* function. Fold changes were calculated as the mean expression values for the two groups of interest and then further log2-transformed. Statistical tests were performed using the R function *t.test* for each comparison by applying an unpaired Student's *t*-test to identify differentially expressed genes. To correct for multiple testing, we calculated the increasing false discovery rates (FDRs) for all four comparisons by label shuffling of the samples in the two groups compared. For the determination of differentially expressed genes, an FDR of 2% was used as cutoff. Furthermore, only transcripts annotated in Ensembl (www.ensembl.org) were included in later analyses.

Heatmaps were generated by reading the normalized and collapsed data into MultiExperiment Viewer (MeV) v 4.4.1 (http://mev.tm4.org). The top differentially expressed genes ($P < 0.0001$, fold change > 2, annotated in Ensembl) identified by the Student's *t*-test were selected and organized by HCL of gene leafs only.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on the subset of significantly differentially expressed genes from each of the three comparative data sets: normal ALCs, LC micronodules and LC/TGCT+hCG compared with FLCs, respectively. GSEA was performed using the DAVID Bioinformatics Package (Huang *et al.*, 2009). We used the functional annotation tool and tested for enrichment among the significantly differentially expressed genes in our comparisons of interest compared with the total genes represented on the array. We were interested in both enriched Gene Ontologies categories and KEGG pathways (Kanehisa *et al.*, 2016) and we considered a Benjamini–Hochberg corrected *P*-value ≤ 0.05 as significant (Benjamini and Hochberg, 1995).

Results

PCA of gene expression profiles of LC populations

The spatial relationship between the gene expression profiles of the different LC populations was assessed by PCA of all LC populations (Fig. 2A). The FLC samples clearly clustered together and were distant

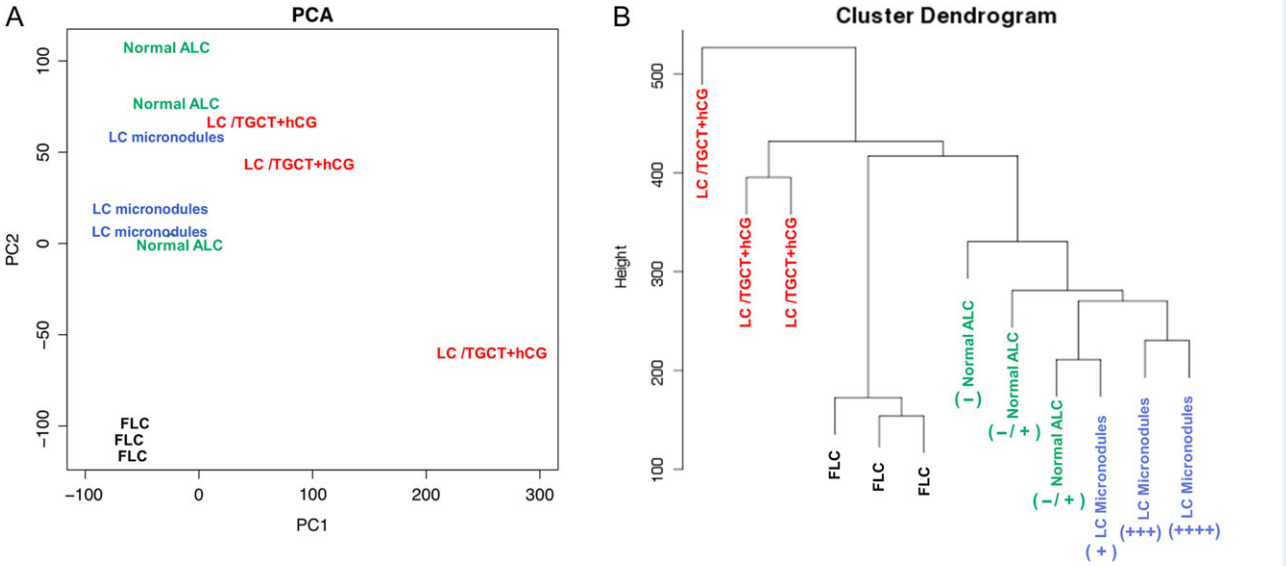

Figure 2 Evaluation of the gene expression data set. Principal component analysis (PCA) of all microdissected LC samples, illustrating the spatial relationship between the samples (**A**). Cluster dendrogram based on hierarchical clustering of all LC samples (**B**). The degree of LC micronodule formation, from no micronodules (−) to many large micronodules (++++), is shown for normal adult Leydig cells (ALCs) and LC micronodules. The samples are distinguished with colours: FLC (foetal LC)—black, Normal ALC—green, LC micronodules—blue, LC/TGCT+hCG (LC isolated from tissue adjacent to hCG-producing testicular germ cell tumours)—red.

from the ALCs. The different ALC populations were less distinguishable; normal ALC and LC micronodules samples clustered together, and the LC/TGCT+hCG group was clearly distinguishable from the other ALC samples. The LC/TGCT+hCG samples showed most variability within the group. The outlier sample of this group also differed in morphology and contained extreme LC hyperplasia with only few seminiferous tubules (data not shown). A similar grouping of the samples was observed in a HCL analysis (Fig. 2B).

Identification of differentially expressed genes

The pairwise differences in global gene expression between the different LC populations were illustrated in 'volcano' plots, which showed the actual differences in gene expression between two categories (coloured black) with the differences that are estimated to occur by chance if the samples were categorized by random (coloured green) (Fig. 3). No differentially expressed transcripts with an estimated FDR ≤ 10% were observed between pairs of any of the ALC categories: LC micronodules versus normal ALC, LC/TGCT+hCG versus normal ALC, and LC micronodules versus LC/TGCT+hCG (Fig. 3A–C). In contrast, the three comparisons including FLCs revealed numerous transcripts with a FDR ≤2% with 968 differentially expressed transcripts ($P \leq 0.002$) observed between normal ALCs versus FLCs (Fig. 3D) 1464 differentially expressed transcripts ($P \leq 0.003$) observed by comparing LC micronodules versus FLCs (Fig. 3E) and 2363 differentially expressed transcripts ($P \leq 0.008$) observed between LC/TGCT+hCG and FLCs (Fig. 3F). Transcripts downregulated in ALCs and thus upregulated in FLCs dominated the top differentially expressed transcripts and represented 64, 63 and 70% of the transcripts in the analyses with normal ALCs, LC micronodules and LC/TGCT+hCG, respectively. If only transcripts with a fold change ≥2 were included, the percentage of transcripts upregulated in FLCs increased to 72, 69 and 83% in the analyses with normal ALCs, LC micronodules and LC/TGCT+hCG, respectively. The lists of the differentially expressed transcripts are available in Supplementary Tables 1–3.

Although no significant differences were observed when comparing the ALC populations to each other, substantial differences were observed in the subsets of genes differentially expressed in the adult categories compared with FLCs (Fig. 4). Only 211 genes were differentially expressed in all categories of ALCs compared with FLCs, and the majority of these genes (92%) were upregulated in the FLCs in comparison with the expression in the three ALC categories. On the other hand, genes differentially expressed between only one of the ALC categories and FLCs were predominantly upregulated in the ALCs; here, the percentages of differentially expressed genes upregulated in ALCs were 75% (normal ALCs), 58% (LC micronodules) and 35% (LC/TGCT+hCG).

GSEA analysis

To assess overrepresented functional categories when comparing ALC populations with FLCs, GSEA was performed on the subset of differentially expressed genes (FDR ≤ 2%) from each of the three pairwise analyses of normal ALCs, LC micronodules or LC/TGCT+hCG versus FLCs, respectively (Fig. 3A–C). All three analyses revealed an overrepresentation of genes involved in mitosis and cell-cycle processes. The results were comparable independent of the ALC category, but more functional categories were significant in the comparison of

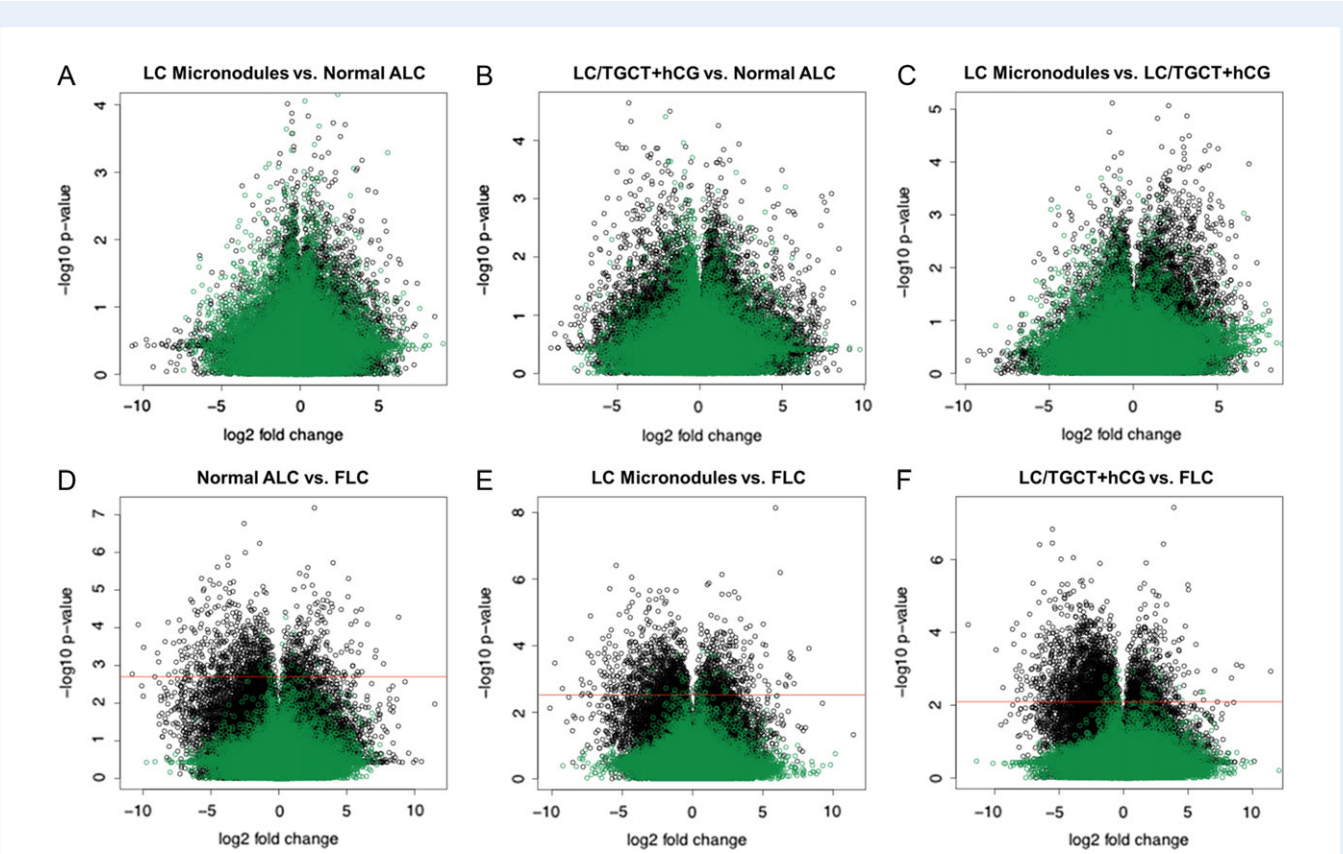

Figure 3 Volcano plots illustrating the differential gene expression between the six categories of specimens. LC micronodules versus Normal ALC (**A**), LC/TGCT+hCG versus Normal ALC (**B**), LC micronodules versus LC/TGCT+hCG (**C**), Normal ALC versus foetal Leydig cell (FLC) (**D**), LC micronodules versus FLC (**E**) and LC/TGCT+hCG versus FLC (**F**). The correct test is shown in black and the random test is shown in green. The red line indicates the false discovery rate = 2%, which was used as the cutoff for further analyses.

LC/TGCT+hCG versus FLCs. The significantly overrepresented categories are listed in Table II, and the complete list of all enriched Gene Ontology categories (Gene Ontology Consortium, 2015) and KEGG (Kanehisa *et al.*, 2016) pathways is available in Supplementary Tables 4–6.

Differential gene expression between adult and foetal LCs

To gain more knowledge of the functional differences between normal ALCs and FLCs, the top differentially expressed genes between these categories were evaluated in greater detail. Although the greatest difference was observed between LC/TGCT+hCG and FLCs, we focussed on the analyses of normal ALCs and FLCs, to exclude possible bias introduced by the testicular pathologies in LC/TGCT+hCG samples. The top differentially expressed genes between normal ALCs and FLCs ($P \leq 0.0001$, fold change ≥ 2) are listed in Table III and illustrated in the heat-map (Fig. 5).

To further validate the transcriptome findings, we chose a subset of differentially expressed genes (Table III) for analysis of expression at the protein level. Four candidate genes, for which good quality antibodies were available, were selected: *SULT2A1* (also known as hydroxysteroid sulfotransferase) and *WISP2* that were upregulated in ALCs compared with FLCs, and *HPGD* and *IGF2BP1* (IMP-1) that were upregulated in FLCs compared with ALCs. The investigation was by IHC in an independent series of tissue samples, including two different stages of foetal development (at 10 and 20–25 gestational weeks) and normal adult testis specimens. As shown in Fig. 6, differential expression patterns were observed.

SULT2A1 protein was detected only in LCs and was expressed in a subset of LCs at all studied ages, but the number of the positive cells and intensity of expression was greater in the adult testis. According to the bioinformatic prediction by GSEA of the cellular localization of this enzyme, the protein was present in the cytoplasm. SULT2A1 is regulated by peroxisome proliferator-activated receptor alpha (Fabregat *et al.*, 2016) and involved in metabolism of steroids and 3′-phosphoadenosine 5′-phosphosulphate (Gene Ontology Consortium, 2015).

WISP2 protein was detected in all studied samples, and was present in the cytoplasm of essentially all cell types in the interstitial tissue, including a subset of LCs, but with a pronounced heterogeneity. The typical polygonal LCs had a lower intensity of WISP2 staining than the more immature cells, except in a couple of samples from the second trimester of pregnancy. WISP2 is a negative regulator of cell death,

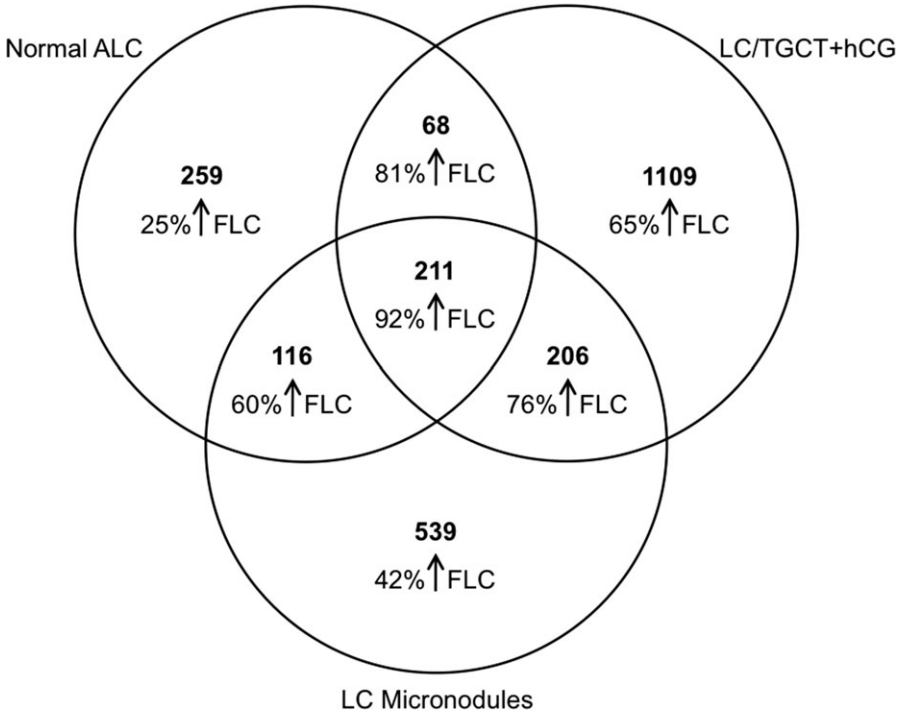

Figure 4 Venn diagram illustrating the overlap in transcripts differentially expressed between the different categories of human ALCs and FLCs. The percentages of transcripts upregulated in FLC compared with ALCs are listed below the number in each category.

regulator of cell growth, involved in cell–cell signalling and signal transduction (Gene Ontology Consortium, 2015).

HPGD protein was detected in all studied testis specimens and was specific for polygonal LCs. However, the staining in foetal samples was much more pronounced than the weak reaction in the adult testes. In the FLCs, the reaction was observed mainly in the cytoplasm, but also in the nucleus in a subset of cells. In the ALCs, the reaction was observed exclusively in the cytoplasm. HPGD is a player in the thrombin and TGF-beta receptor pathways, involved in the metabolism of prostaglandins and lipoxin, and a negative regulator of the cell cycle (Gene Ontology Consortium, 2015).

IMP-1, a protein encoded by *IGF2BP1*, was detected in all studied specimens but with marked heterogeneity of the expression pattern depending on the age. At the early foetal time point (GW 10), a strong reaction was observed in the entire interstitial tissue, with additional weak reaction in Sertoli cells, but no reaction was detected in germ cells. By contrast, in the later foetal stage (GW20–25) no or very weak staining was observed in LCs but a clear reaction was detected in the cytoplasm of germ cells. The same pattern was also observed in the adult testis specimens, where the germ cell staining was limited to spermatogonia. IMP-1 is a regulator of mRNA translation, localization and stability (Gene Ontology Consortium, 2015).

All associated Gene Ontology biological process categories, KEGG and Reactome pathways of the four proteins mentioned above are listed in Supplementary Table 7. In addition, to facilitate searches for genes of interest, the complete data from the arrays listed according to the gene name are presented in Supplementary Table 8, and have been deposited in a public depository ArrayExpress (accession code E-MTAB-5453).

Discussion

This is the first reported comparative study of global gene expression profiles of microdissected human LCs at different stages of development. The study identified a number of novel markers specific for either FLCs or ALCs and demonstrated that hyperplastic LCs within micronodules exhibited the adult gene expression 'signature'. The observed profiles speak against the hypothesized similarities of LCs within the LC micronodules and FLCs.

Our initial hypothesis was that the LCs clustered in large micronodules in disorders associated with TDS, in particular in patients with germ cell neoplasia, might have a different expression profile. Surprisingly, no significant differences in the gene expression profiles were observed between normally clustered ALCs and LCs dissected from micronodules. We had expected some differences, as the LC populations in such patients contain an increased proportion of progenitor and immature LCs (distinguished by high expression of DLK1), as a consequence of the increased drive for ALC renewal mediated by high hCG levels (Lottrup *et al.*, 2014). Although no significant differences between the different ALC populations were identified, some trends were observed in the cluster analysis; the LC/TGCT+hCG samples did cluster separately from the other ALC populations (as shown in Fig. 2B). Hence, we believe that there are discrete changes in the global gene expression associated with the presence and severity of LC micronodules, but that these changes at the level of single genes are too small to be detected in this analysis.

The lack of significant differences between the different ALC populations observed in this study might imply that the observed functional

Table II Significant categories from the gene set enrichment analysis performed for each subset of differentially expressed transcripts between the human FLCs and Normal ALCs or hCG-stimulated adult LCs (LC/TGCT+hCG).

Analysis	Category*	Term	Count	%	*P*-value	Fold enrichment	Benjamini *P*-value
Normal ALC	BP	Cell cycle	51	8.1	0	1.9	0.028
		Spindle organization	10	1.6	0	6.3	0.05
LC/TGCT+hCG	BP	Cell-cycle phase	68	4.4	0	1.9	0.001
		DNA metabolic process	77	5.0	0	1.8	0.001
		Mitotic cell cycle	60	3.9	0	1.9	0.002
		Cell cycle	104	6.7	0	1.6	0.003
		Cell-cycle process	80	5.2	0	1.7	0.005
		Organelle fission	41	2.6	0	2.1	0.006
		M phase	52	3.4	0	1.8	0.01
		RNA processing	76	4.9	0	1.6	0.011
		Nuclear division	38	2.5	0	2.0	0.018
		Mitosis	38	2.5	0	2.0	0.018
		M phase of mitotic cell cycle	38	2.5	0	2.0	0.024
		Cellular response to stress	76	4.9	0	1.6	0.024
		DNA replication	33	2.1	0	2.0	0.041
	CC	Nuclear lumen	179	11.5	0	1.6	0
		Intracellular organelle lumen	210	13.5	0	1.5	0
		Organelle lumen	213	13.7	0	1.5	0
		Membrane-enclosed lumen	216	13.9	0	1.5	0
		Nucleoplasm	121	7.8	0	1.7	0
		Non-membrane-bounded organelle	263	17.0	0	1.3	0.001
		Intracellular non-membrane-bounded organelle	263	17.0	0	1.3	0.001
		Chromosomal part	57	3.7	0	1.9	0.001
		Chromosome	64	4.1	0	1.8	0.001
		Collagen	11	0.7	0	4.0	0.015
		Spliceosome	24	1.5	0	2.3	0.016
		Microtubule cytoskeleton	67	4.3	0	1.5	0.021
		Spindle	25	1.6	0.001	2.1	0.023
		Chromatin	31	2.0	0.001	2.0	0.024
		Microtubule	38	2.5	0.001	1.7	0.036
		Ribonucleoprotein complex	62	4.0	0.001	1.5	0.038
	MF	RNA binding	95	6.1	0	1.6	0.008
		Nucleotide binding	245	15.8	0	1.3	0.014

*BP, Biological process; CC, cellular compartment; MF, molecular function.

impairment is caused by post-transcriptional or post-translational alterations affecting only protein functions. We did not specifically investigate such modifications, so this is a hypothesis that would require further studies to corroborate. Another likely explanation is a general problem of high intra-group variation. The study was constrained by the lack of availability of truly normal control testis samples. The material investigated in this study was all derived from orchiectomy specimens, and although the morphology appeared normal in the sections used to isolate the 'normal' ALCs analysed in this study, the testis as a whole may not be regarded as normal due to the presence of GCNIS or TGCTs. The inclusion of a better control group with samples from testis without tumours, e.g. biopsies from men with obstructive forms of azoospermia, might have improved the study.

The most interesting results of this study concern the expression profiling of human foetal testis samples isolated from embryos at the prenatal age of GW 11–13. Human FLCs clearly clustered separately from all categories of ALCs, and their global gene expression profiles revealed marked overrepresentation of upregulated genes when compared with the ALCs. The greatest differences were observed between FLCs and the LCs from testes with hCG-producing tumours. This surprising pattern could reflect a different response of FLCs and ALCs to high hCG stimulation, as observed decades ago in rats. While the rat FLCs respond with increased steroidogenesis, the ALCs can become insensitive to further stimulation by down-regulation of LH receptors or key enzymes in steroidogenesis (Dufau, 1988; Huhtaniemi and Pelliniemi, 1992).

Table III Transcripts differentially expressed between human normal ALCs and FLCs.

Symbol	FC*	*P*-value	Possible function**	Symbol	FC	*P*-value	Possible function
Transcripts upregulated in FLCs compared with normal ALCs							
SEMA4G	−3.8	1.36E-06	Axon guidance	TYMS	−5.0	3.83E-05	DNA replication
							DNA repair
ASTN1	−3.7	2.17E-06	Neuronal adhesion	SCG5	−3.1	3.84E-05	Hormone secretion
PXDN	−4.5	2.45E-06	Peroxidase activity	PHLDA1	−3.4	4.02E-05	Regulation of apoptosis
ADRA2C	−5.7	4.89E-06	Neurotransmission	ZNF114	−2.9	4.14E-05	Transcriptional regulation
GPX7	−5.1	5.45E-06	Cellular stress response	ADAMTS14	−5.2	4.15E-05	Aminoprocollagen peptidase
ODZ4	−4.4	6.79E-06	CNS formation	ADAMTS9	−5.2	4.44E-05	Organ shape
SORBS1	−4.8	7.27E-06	Glucose transport	RECK	−6.0	4.53E-05	Neg. regulator of metallo-proteinase-9
CXCL12	−5.5	9.24E-06	Chemo-attractant	HNRNPCL1	−3.2	5.09E-05	Nucleosome assembly
IGF2BP1***	−5.6	1.03E-05	Binding protein	KALRN	−2.9	5.25E-05	Neuronal growth
NYNRIN	−3.6	1.18E-05	-	ARHGAP11A	−3.0	5.91E-05	Rho GTPase Activation
HPGD***	−6.5	1.79E-05	Prostaglandin metabolism	HBG1	−7.3	6.19E-05	Foetal haemoglobin F
GPR183	−3.6	2.02E-05	B-cell maturation	MARCKS	−3.8	6.31E-05	Cell motility
							Mitogenesis
PTCH1	−2.1	2.06E-05	Foetal LC development	SF3B3	−2.3	6.48E-05	Splicing
							DNA repair
CDKN1C	−3.4	2.22E-05	Proliferation	IQCJ-SCHIP1	−4.5	7.62E-05	Ca^{+2} mediated responses
OXCT1	−3.2	2.39E-05	Ketone body catabolism	FLRT3	−5.7	7.83E-05	Cell adhesion
							Receptor signal
C15orf42	−3.7	2.42E-05	DNA replication	KIAA0101	−10.3	8.28E-05	DNA repair
PDS5B	−3.0	2.54E-05	Mitosis	CTTNBP2NL	−2.1	8.49E-05	
KIF18A	−5.3	2.60E-05	Mitosis	PSIP1	−2.3	8.60E-05	Neurogenesis
C12orf48	−4.7	2.89E-05	DNA repair	IGSF3	−3.8	8.88E-05	
CDT1	−4.0	3.05E-05	DNA replication	FERMT1	−2.6	8.95E-05	Cell adhesion
							Integrin activity
CACNA1G	−5.7	3.34E-05	Calcium channel	SH3RF1	−3.2	9.01E-05	Apoptosis
CXCR4	−6.0	3.79E-05	Angiogenesis	DTL	−7.0	9.66E-05	Cell cycle
			Apoptosis				
Transcripts upregulated in normal ALCs compared with FLCs							
SMG9	2.6	6.57E-08	Post-transcriptional regulation	TSPAN8	6.1	3.25E-05	Cell development
CLTB	2.1	2.53E-06	Endocytosis	SULT2A1***	4.6	3.26E-05	Steroid sulphonation
MOB3A	2.0	4.15E-06	Kinase activity	TMEM219	2.9	3.77E-05	Apoptosis
WISP2***	5.1	4.95E-06	Bone turnover	LGALS3	3.7	3.81E-05	Apoptosis
							Cell adhesion
MSI2	2.5	7.44E-06	Post-transcriptional regulation	AKR1C1	2.8	4.49E-05	Progesterone inactivation

SLC25A18	2.6	1.24E-05	Glutamate transportation	TREX1	3.1	5.10E-05	DNA repair
C9orf128	3.6	1.26E-05	-	PRM1	8.8	5.25E-05	Sperm DNA condensation
NUPR1	5.1	1.50E-05	Cellular stress resistance	SSSCA1	2.2	6.00E-05	Mitosis
MGLL	3.2	1.84E-05	Fatty acid metabolism	CBX7	3.2	6.08E-05	Transcriptional repression
SPDYA	3.9	2.28E-05	Cell cycle regulation	C1orf111	3.1	6.38E-05	
PDXK	2.7	2.35E-05	Vitamin B6 metabolism	MGP	5.7	6.69E-05	Bone formation regulation
SYNGR1	3.2	2.79E-05	Synaptic plasticity	SPEM1	3.4	7.38E-05	Spermatogenesis
CMTM1	2.2	2.82E-05	Testis development	FBP1	2.9	7.48E-05	Glucogenesis
ACRBP	3.9	2.83E-05	Acrosomal formation	ALDH1L1	6.5	8.02E-05	Aldehyde de-hydrogenation
HP	3.3	2.90E-05	Haemoglobin clearance	DUS1L	2.1	8.17E-05	

*FC, fold change.
A more comprehensive information on possible functions for each transcript is found in Supplementary Table 4 and for transcripts studied at the protein level (marked with *) in Supplementary Table 7, which list all Gene Ontology categories from the gene set enrichment analysis performed using DAVID on subset of differentially expressed genes between normal ALC and FLC ($P = 0.001$, false discovery rate = 2%).
***Selected for validation at the protein level by immunocytochemistry. The protein expression pattern of these factors in testicular specimens is shown in Fig. 6.

When assessing functional categories by GSEA, genes involved in mitosis, DNA replication, DNA repair and other cell-cycle processes were clearly overrepresented in FLC, consistent with increased cell proliferation. A study in mice reported that proliferating cells in the foetal interstitial compartment were predominantly of mesenchymal origin, whereas a distinct wave of LC proliferation was demonstrated at puberty (Vergouwen *et al.*, 1991). In our data, the presence of mesenchymal cells located between the LCs in the foetal gonad would likely contribute to the observed signal, but mitotic activity of human FLCs needs further investigations to be proven. In the adult, mitotic LCs are very rarely seen, as renewal of the LC population mainly occurs through recruitment of cells from earlier stages of differentiation. Increased numbers of mitotic LCs have been reported only in adult patients with TGCTs (Lauke *et al.*, 1989), and in large micronodules associated with hCG-producing tumours (Lottrup *et al.*, 2014).

Among the top 75 genes differentially expressed between FLCs and ALCs in this study, a number of interesting candidates were identified. Several genes upregulated in FLCs were coding for proteins with a known function in the nervous system, including neurogenesis and neurotransmission, such as adrenoceptor alpha 2C (*ADRA2C*), astrotactin 1 (*ASTN1*), kalirin (*KALRN*), PC4 and serine and arginine rich splicing factor 1 (SFRS1)-interacting protein 1 (*PSIP1*). This is in concert with the reported enrichment of genes involved in neuroactive signalling in murine FLCs (McClelland *et al.*, 2015). PSIP1, known to be also involved in lens development and an anti-apoptotic factor, was recently described in epithelial ovarian cancer cell lines (French *et al.*, 2016). This and other neurogenic factors, e.g. *ASTN1* or *KALRN*, are listed in public databases as associated with developing brain and spine tissue, but have not been previously detected in the testis, so their possible involvement in the function of foetal mesenchymal or LCs remains to be corroborated.

By contrast, the top genes upregulated in ALCs included genes coding for proteins involved in post-transcriptional regulation or processing, with known functions in bone and spermatogenesis. The detection of genes involved in spermatogenesis was most likely a sign of some germ cell material admixed in the ALC RNA, and emphasized the need for a thorough validation of the findings. It is exceedingly difficult while working on frozen sections to dissect pure cells and there is nearly always some leakage from the neighbouring cells. However, it was reassuring for the data set that known LC-specific genes including *INSL3* and *HSD3B1* were detected at high levels of expression in all investigated adult samples (shown in Supplementary Table 8).

The link to regulation of bone growth and metabolism has been increasingly evident in LC physiology (Ferlin *et al.*, 2013). A high expression in ALCs of two bone-turnover associated genes, matrix Gla protein (*MGP*) and *WISP2*, is thus very interesting. MGP protein is a vitamin K-dependent member of the osteocalcin/matrix Gla family, known to be secreted by chondrocytes and vascular smooth muscle cells, and to inhibit calcification of soft tissues (Luo *et al.*, 1997). Vitamin K insufficiency has been shown to impair testicular steroideogenesis in rats and this was attributed to decreased expression of Cyp11a (Shirakawa *et al.*, 2006), but further studies are warranted to clarify whether or not MGP is involved in human LC.

Another gene upregulated in ALCs with a link to bone was *WISP2*, also known as *CCN5*. This gene is, at the protein level, quite broadly expressed in endocrine organs (including adipose tissue) and connective tissue. Particularly, high levels of WISP2 protein were reported in

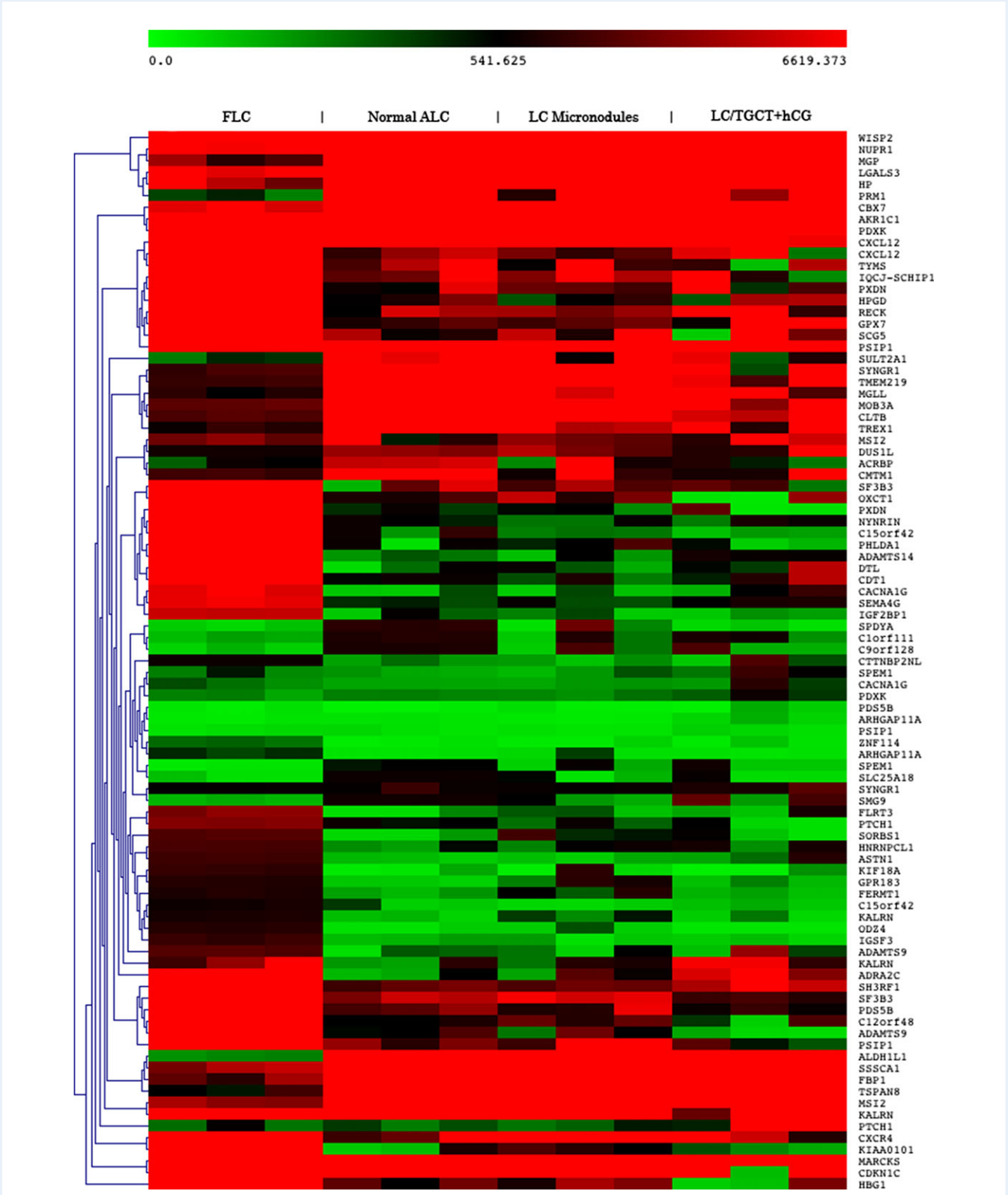

Figure 5 Heatmap of top 75 differentially expressed genes in human LCs. ($P < 0.0001$, fold change > 2, software R *corPvalueStudent*) between four LC populations (from the left): FLCs, normal ALCs, LC micronodules and LC/TGCT+hCG. The horizontal bar on top of the figure shows the range of colours used to mark relative levels of gene expression: green colour depicts low expression and red colour a high expression, with black marking the moderate levels in between.

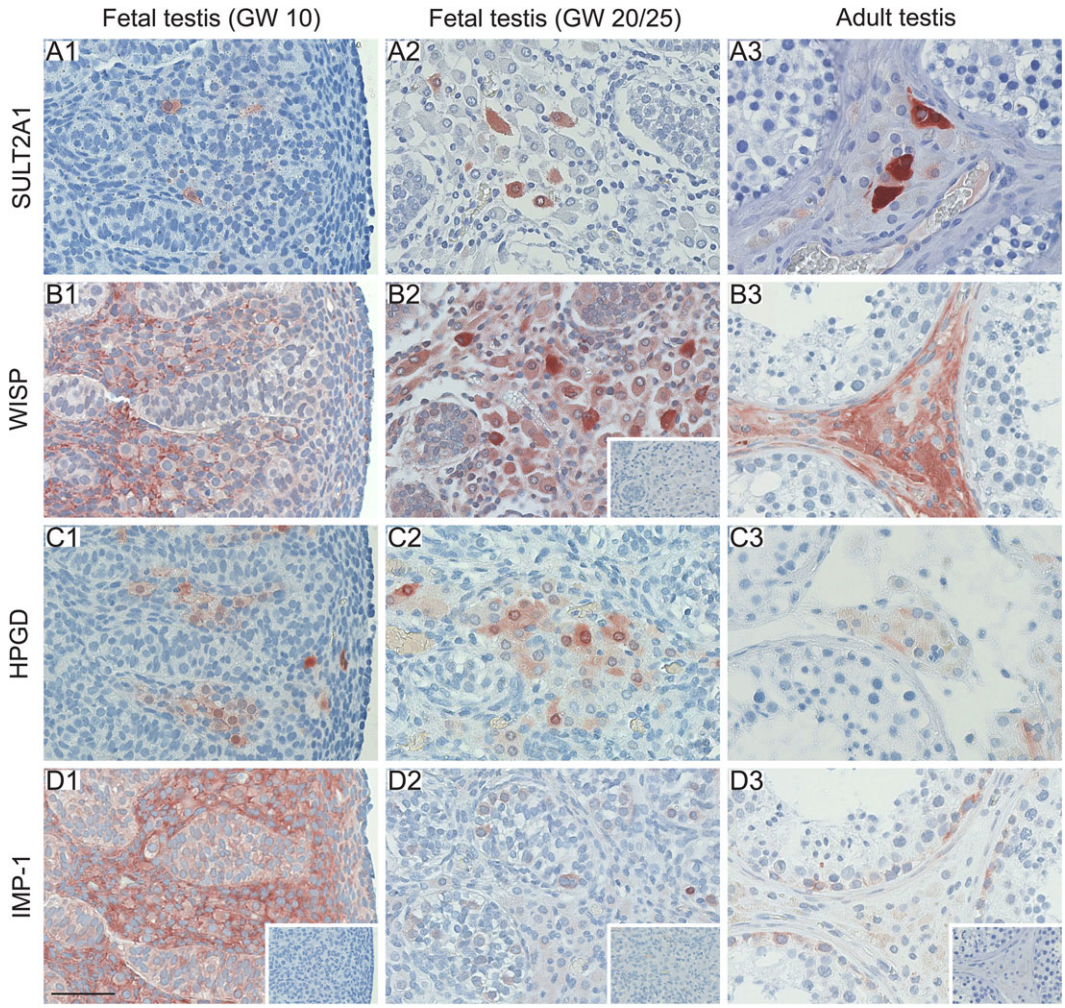

Figure 6 Validation of array data at the protein level by IHC. Protein expression of sulfotransferase family 2A member 1, SULT2A1 (A1–3), WNT1-inducible signalling pathway protein 2, WISP (B1–3), hydroxyprostaglandin dehydrogenase, HPGD (C1–3) and insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), IMP-1 (D1–3) in foetal testis specimens at first trimester (left column), gestational week (GW) 10 and second trimester (middle column) GW20 (B2, D2) or GW25 (A2, C2), and in adult testis specimens (right column). All pictures have the same magnification and the bar in D1 (left bottom corner) corresponds to 50 µm. Small insets in 4-fold lower magnification visible in corners of several images show negative controls in the same specimens.

bone tissue, suggesting that this protein is involved in modulating bone turnover (Kumar *et al.*, 1999). WISP2 has not been described previously in human LCs but was reported to be expressed in the rodent testis (Gray *et al.*, 2007) and in human testis tissue with or without pre-invasive germ cell neoplasia (Skotheim *et al.*, 2005). Thus, we have selected this gene for validation at the protein level and showed in this study that WISP2 was present in essentially all mesenchymal cells within the interstitial compartment. The marked heterogeneity of the protein expression in LCs, especially in the adult testis, is intriguing and warrants further functional exploration.

In addition to *WISP2*, several additional functionally interesting differentially expressed transcripts were validated at the protein level. An interesting observation was the differential expression pattern of IGF2BP1/IMP-1. This protein belongs to a family of IGF-mRNA binding proteins and was reported to bind predominantly IGF2 transcripts, and to regulate proliferation of mesenchymal stem cells (Mahaira *et al.*, 2014). We found in this study that during early embryogenesis IMP-1 is highly expressed in all mesenchymal cells and in Sertoli cells, but the expression in FLCs is apparently rapidly downregulated already in the second trimester of gestation and the protein becomes largely restricted to spermatogonia. Such a switch of the expression from a somatic cell to a germ cell is uncommon, but this finding is supported by a previous study which detected IMP-1 in human testis tissue using a different antibody (Hammer *et al.*, 2005).

Among the genes that at the transcript level were expressed more strongly in ALCs than in FLC, was *SULT2A1*. This gene codes for steroid sulfotransferase, an enzyme involved in sulphate conjugation, for

example of dehydroepiandrosterone or pregnenolone, primarily in adrenal glands and the liver, but also active in detoxification of xenobiotics (Rainey and Nakamura, 2008; Squirewell *et al.*, 2014). Expression of *SULT2A1* was described in pig testicles (Grindflek *et al.*, 2010), but to the best of our knowledge, our study is the first demonstration of SULT2A1 protein in human LCs. Foetal expression of *SULT2A1* at the transcript level has been reported in adrenals, liver and kidneys but not in testicles (O'Shaughnessy *et al.*, 2013; Ekström and Rane, 2015). Immunohistochemical staining revealed that SULT2A1 was expressed only in a subset of cells in the interstitial compartment. The rare SULT2A-positive cells present in the foetal testis specimens could perhaps be adrenal precursor cells, considered the cell of origin of testicular adrenal rest tumours found in males with congenital adrenal hyperplasia (Lottrup *et al.*, 2015). However, the cells marked by abundant SULT2A1 staining in the adult testis had a very typical ALC morphology (Fig. 6). Hence this finding needs further validation and analysis in a larger number of samples. Analysis of the expression of other genes involved in steroidogenesis, especially those involved in mitochondrial stages, did not reveal significant changes between FLCs and ALCs.

In conclusion, the transcriptome data obtained in this study provide a valuable resource for exploring the functional differences between FLCs and ALCs. The different gene expression profiles revealed potential markers of developmental stage-specific functions, and functional evaluation of these potential candidates certainly warrants further studies. On the other hand, despite morphological differences between normal ALCs and LCs within micronodules present in disorders associated with testicular dysgenesis, no significant differences in global gene expression were observed between the two groups. However, there are likely differences at the single-cell level in LCs within heterogeneous micronodules, especially those in the vicinity of hCG-producing tumours. The impairment of LC function in patients with reproductive disorders is likely to be caused by subtle changes in the expression of many genes rather than by failure of normal regulation of single genes or pathways.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

G.L., H.L., S.B., N.E.S., A.J. and E.R.-D.M. designed and co-supervised the study. G.L., J.E.N. and M.D.D. performed the experiments. K.B. performed the bioinformatic analysis. G.L., K.B., H.L. and E.R.-D.M. analysed the data and results. G.L., K.B. and E.R.-D.M. wrote the manuscript. All authors contributed to writing and approved the text.

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Conflict of interest

None declared.

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