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# ORIGINAL ARTICLE

# Transcriptome profiling of fetal Klinefelter testis tissue reveals a possible involvement of long non-coding RNAs in gonocyte maturation

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## Abstract

In humans, the most common sex chromosomal disorder is Klinefelter syndrome (KS), caused by the presence of one or more extra X-chromosomes. KS patients display a varying adult phenotype but usually present with azoospermia due to testicular degeneration, which accelerates at puberty. The timing of the germ cell loss and whether it is caused by dysgenetic fetal development of the testes is not known. We investigated eight fetal KS testes and found a marked reduction in MAGE-A4-positive pre-spermatogonia compared with testes from 15 age-matched controls, indicating a failure of the gonocytes to differentiate into pre-spermatogonia. Transcriptome analysis by RNA-sequencing of formalin-fixed, paraffin-embedded testes originating from four fetal KS and five age-matched controls revealed 211 differentially expressed transcripts in the fetal KS testis. We found a significant enrichment of upregulated X-chromosomal transcripts and validated the expression of the pseudoautosomal region 1 (PAR1) gene, *AKAP17A*. Moreover, we found enrichment of long non-coding RNAs in the KS testes (e.g. *LINC01569* and *RP11–485F13.1*). In conclusion, our data indicate that the testicular phenotype observed among adult men with KS is initiated already in fetal life by failure of the gonocyte differentiation into pre-spermatogonia, which could be due to aberrant expression of long non-coding RNAs.

## Introduction

Klinefelter syndrome (KS) is the most common sex chromosomal disorder estimated to occur in 1 out of 660 newborn boys (1). The most prevalent karyotype is 47, XXY, but also higher degrees of aneuploidies (e.g. 48, XXYY and 49, XXXXY) as well as various degrees of mosaicisms have been described (2). The adult man with KS has varying symptoms including increased height, gynecomastia, hypergonadotropic hypogonadism with serum testosterone in low-normal range and elevated levels of LH and FSH, and small testes (3,4). Due to the variable and, sometimes, very mild phenotype, the syndrome is highly under-diagnosed with less than 10% of the expected cases being diagnosed before puberty and only 25% diagnosed throughout life (1). One of the most consistent problems in men with non-mosaic KS is infertility. These men are usually azoospermic or

severely oligozoospermic, and the testicular histology shows high amounts of degenerated, hyalinized tubules and a complete lack of germ cells besides reduced amounts of Sertoli cells and pronounced Leydig cell hyperplasia (2,3). Consequently, having an additional X-chromosome seems quite incompatible with normal spermatogenesis.

It is the general perception that testicular development in KS boys is quite normal until puberty. However, at puberty the induction of spermatogenesis fails in the KS testis, which leads to a rapid testicular degeneration in young adulthood (2). The existing evidence concerning the beginning of the germ cell loss is scarce and contradictory. Indeed, two studies reported no differences in germ cell numbers between 47, XXY KS fetuses at gestational week (Gw) 20 and 22 compared with age-matched controls albeit no actual quantification of germ cells was made (5,6). In contrast, one study reported that the amount and volume of germ cells in 47, XXY KS fetuses aged Gw18 to 22 were markedly reduced compared with age-matched controls (7). Moreover, two studies reported a highly reduced number of germ cells in the testes of 49, XXXXY KS fetuses (8,9).

The molecular mechanisms behind the KS phenotype are poorly understood.

 Several recent studies have investigated the transcriptome of KS patients compared with controls. In four studies, transcriptome analysis was carried out on RNA isolated from whole blood. Three of the studies investigated adult KS men (10–12), whereas one study included a total of 8 KS patients aged 3 months to 26 years (13). The only recurring finding is upregulation of the long non-coding RNA (lncRNA) *XIST* in KS men compared with controls (10–13). This is expected, since *XIST* is the major RNA involved in X-chromosome inactivation (XCI) ensuring dose compensation when more than one X-chromosome is present (14,15). Three of the studies, in addition, found upregulation of *GTPBP6* (11–13), which is confined to the pseudoautosomal region 1 (PAR1) on the X-chromosome. Other upregulated transcripts reported by at least two of the studies, included PAR1-transcripts *AKAP17A*, *CSF2RA*, *SLC25A6* and *ASMTL*, the Xp-transcripts *KDM5C*, *ZFX*, *PRKX*, *EIF2S3* and *EIF1AX*, and the Xq-transcript *RPS4X*. Only one downregulated transcript, *LAMB2*, which is expressed from chromosome 3, was detected by more than one study (11–13).

Recently, D'Aurora *et al.* published two microarray studies of gene expression patterns in the adult KS testis. One study compared amplified RNA from testicular biopsies from six non-mosaic azoospermic KS patients to a pool of three biopsies with normal spermatogenesis (16). While this is a unique study of material that is very hard to obtain, it is hampered by the huge cellular differences between the two groups leading to "false" identification of differentially expressed transcripts (DETs). In the second study, analysis of amplified RNA from three adult KS samples with still ongoing spermatogenesis was reported (17). Even though probes for *XIST* were on the microarray, these studies did not report upregulation of *XIST*.

While it is central to understand how the extra X-chromosome affects adult spermatogenesis, it is equally important to discriminate between effects on spermatogenesis and effects caused by improper development of the testis. In this study, we addressed these gaps of knowledge and investigated a series of archival samples of fetal KS testis and age-matched controls by transcriptome analysis and immunohistochemical (IHC) studies of germ cells, with careful consideration for differences in distribution of cell types (cellularity). We found changes in the germ cell differentiation pattern and identified several candidate genes, including ncRNAs. Collectively the data presented here provide novel evidence for the developmental origin of the testicular phenotype in KS.

## Results

### Developmental expression of germ cell markers

To evaluate if germ cell loss begins during fetal life in KS, a total of 8 KS testes from second trimester as well as 15 age-matched controls were stained with the gonocyte marker OCT3/4 and the pre-spermatogonia marker MAGE-A4 (Fig. 1A). The number of OCT3/4-positive cells decreased from Gw13 to 22, which was evident for both control and KS fetuses (Fig. 1B). On the other hand, a clear increase in the number of MAGE-A4-positive cells was observed in the controls, but not in the KS fetuses. Moreover, two KS fetuses with the karyotype 49, XXXXY had very few OCT3/4- and MAGE-A4-positive cells already at Gw14 and 15 (Fig. 1B). This is in agreement with previous studies (8,9) and underlines the severity of the 49, XXXXY syndrome. We found no differences in the interstitial area per total area (Fig. 1B).

### Transcriptome analysis

We next performed global transcriptome analysis of fetal testis samples. A total of 14 formalin-fixed, paraffin-embedded (FFPE) testis samples were included in this study (Table 1). In general, the RNA quality was compromised due to FFPE treatment and long-term storage and resulted in an average of 1.7 million reads per sample. Quality control indicated that three samples, fKS6, fNorm2, and fNorm7, appeared very different from the rest of the samples (Fig. 2 and Supplementary Material, Fig. S1) and were therefore excluded. From previous studies (10–13), we know that *XIST* should be upregulated in KS and we consequently did a sanity check for *XIST* expression among the KS samples. Four of the remaining six samples expressed *XIST* at a higher level than the controls, and only these samples were included in the final analysis (Fig. 2 and Supplementary Material, Fig. S1C).

Using an unadjusted *P*-value of less than 0.01 and an absolute log$_2$ fold change (logFC)$\geq$1.5 or $\leq$1.5, a total of 211 DETs were identified, with 153 being upregulated and 58 being downregulated in the fetal KS samples compared with the controls (Fig. 3A and B and Supplementary Material, Tables S1 and S2). To evaluate if the differential expression was merely due to differences in cellular composition between KS testes and control testes, we did a sanity check for expression of known Sertoli cell, peritubular cell and Leydig cell markers and found no systematic differences between the groups (Supplementary Material, Fig. S2).

Apart from *XIST*, 16 X-chromosomal transcripts were upregulated (Fig. 3C and Supplementary Material, Table S1), including two from the PAR1 region: *AKAP17A* and ENSG00000275287 (*Metazoa_SRP*). Among other X-chromosomal transcripts, *NLGN4X* has been reported to escape XCI and *CD40LG* has shown variable patterns of escape from XCI [XCI status according to Balaton *et al.* (18)]. The remaining transcripts have no or discordant data regarding XCI status. No X-chromosomal transcripts were downregulated.

### Validation by RT-qPCR

Using amplified RNA, we were able to validate the upregulation of *AKAP17A* in two fetal KS testes (fKS2 and fKS3) and two controls testes (fNorm3 and fNorm4) by RT-qPCR (Fig. 4). We also attempted to validate *AWAT2* and *MLLT3* but probably due to
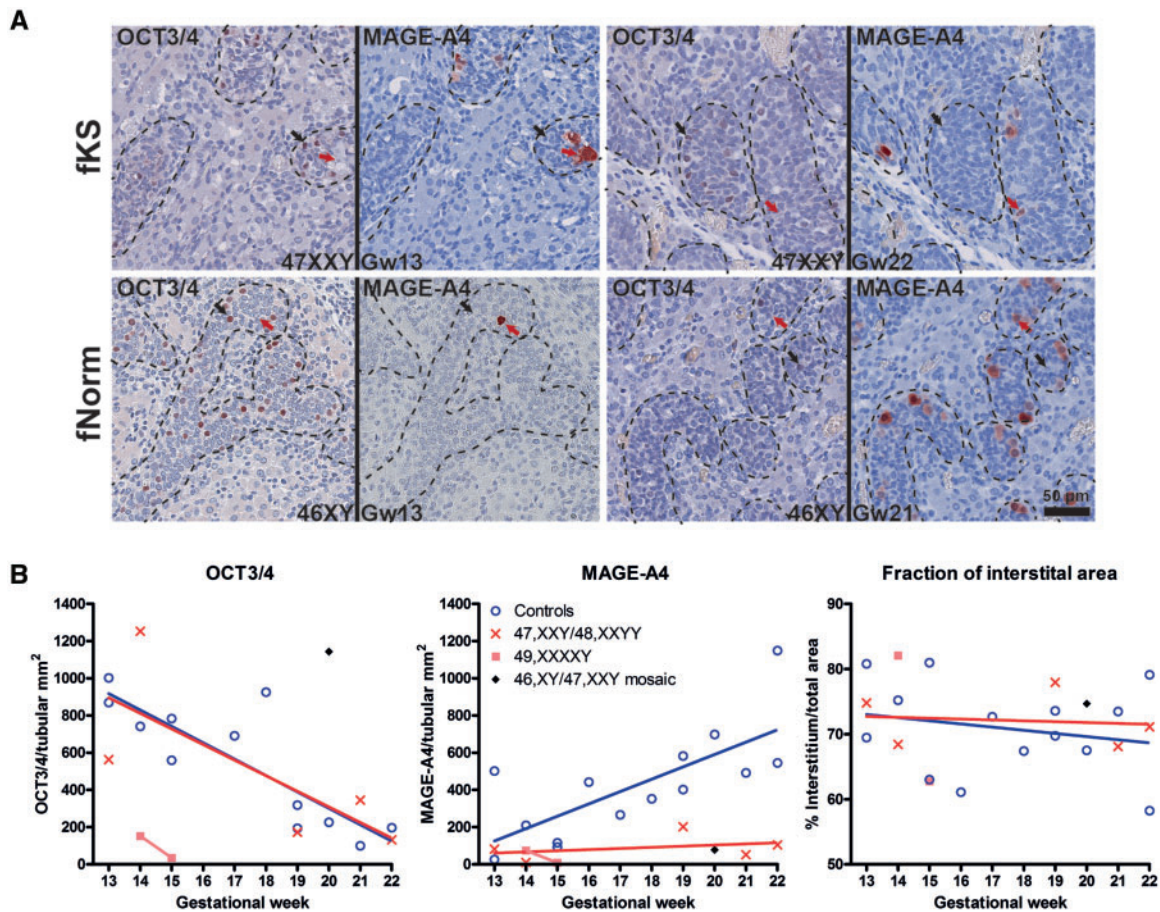
**Figure 1.** Cellularity of the samples. (**A**) Examples of immunohistochemical (IHC) staining (serial sections) of testes from fetuses with Klinefelter syndrome (KS) aged gestational week (Gw) 13 and 22 ($n = 8$), and age-matched controls ($n = 15$). Black arrows indicate OCT3/4-positive cells, whereas red arrows indicate MAGE-A4-positive cells. The tubular edges are shown with dotted lines. (**B**) Quantification of OCT3/4-positive gonocytes (left) and MAGE-A4-positive pre-spermatogonia (middle) per tubular area in mm². Fraction of interstitial area per tubular area (right). The linear fits are indicated with lines.
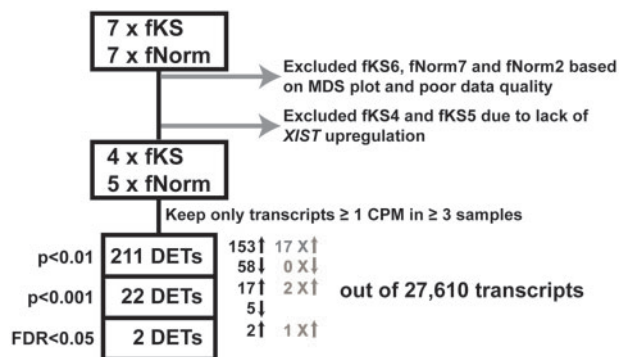


**Figure 2.** Flowchart of the initial RNA-Seq data analysis. fKS: fetal Klinefelter syndrome, fNorm: fetal normal, CPM: counts per million, DETs: differentially expressed transcripts.

missing amplification, the qPCR probes did not produce useful data.

### Enrichment analysis

Analysis of the number of DETs per chromosome, revealed a significant enrichment (P-value = 0.0098) of transcripts expressed from the X-chromosome (Figs 3C and 5A). A total of 963 X-

chromosomal transcripts were expressed in the dataset and a barcode plot revealed an enrichment of upregulated transcripts (Fig. 5B). We also searched for enrichment of DETs in blood from KS compared with controls. Of the 13 transcripts that overlap between at least two previous studies (11–13), we found a significant (P-value = 9.1E-10) upregulation of these transcripts in an enrichment analysis (Fig. 5C).

We noticed that 136 of the 211 DETs were non-protein-coding (Supplementary Material, Tables S1 and S2). We therefore analysed the biotype of the transcripts as classified by the GENCODE annotation (19). This revealed that many of the transcripts were long, intergenic ncRNAs (lincRNAs). When we compared the fraction of identified lincRNAs among all DETs to the fraction of all lincRNAs in our dataset among all detected transcripts, we found a significant (P-value = 0.01) enrichment of lincRNAs (Fig. 5D and Supplementary Material, Table S3). Also, transcripts categorized as antisense (P-value = 0.03), sense intronic (P-value = 0.004) and TEC (P-value = 0.04, TEC are potential novel single exon genes To be Experimentally Confirmed) were enriched (Fig. 5D and Supplementary Material, Table S3).

## Discussion

Little is known about the development of the KS testis and what the functional consequences the extra X-chromosome during

**Table 1.** Fetal samples

| ID (RNA-Seq) | Group | Age of patient (wg) | Karyotype | Age of block[a] in years | Library size[b] |
|---|---|---|---|---|---|
| fKS1 | Fetal KS | 13 | 46XY(3)/47XXY(57) | 23 | 1 602 726 |
| fKS2 | Fetal KS | 14 | 49XXXXY(81)/48XXXY(14)/47XXY(5) | 6 | 2 899 231 |
| fKS3 | Fetal KS | 14 | 47XXY | 3 | 1 611 621 |
| fKS4 | Fetal KS | 15 | 49XXXXY | 5 | |
| fKS5 | Fetal KS | 19 | 47XXY | 6 | |
| | Fetal KS | 20 | 46XY/47XXY ca. 50% | 8 | |
| fKS6 | Fetal KS | 21 | 48XXYY | 21 | |
| fKS7 | Fetal KS | 22 | 47XXY | 3 | 1 248 990 |
| fNorm1 | Fetal control | 13 | | 9 | 413 191 |
| | Fetal control | 13 | | 4 | |
| fNorm2 | Fetal control | 14 | | 8 | |
| fNorm3 | Fetal control | 15 | | 6 | 2 416 077 |
| | Fetal control | 15 | | 4 | |
| | Fetal control | 16 | | 10 | |
| fNorm4 | Fetal control | 17 | | 4 | 2 098 532 |
| | Fetal control | 18 | | 10 | |
| fNorm5 | Fetal control | 19 | | 18 | 565 885 |
| | Fetal control | 19 | | 20 | |
| | Fetal control | 20 | | 10 | |
| | Fetal control | 20 | | 16 | |
| fNorm6 | Fetal control | 21 | | 9 | 2 011 831 |
| fNorm7 | Fetal control | 22 | | 18 | |
| | Fetal control | 22 | | 22 | |

[a]Age of block refers to the age at the time the RNA was extracted.
[b]Only samples with an indicated library size were used for RNA-seq.
Abbreviations: KS: Klinefelter syndrome; wg: weeks of gestation.

this period are. A characteristic phenotype of the KS testis is the degeneration and hyalinization of the seminiferous tubules that occur at puberty (3). Based on few samples it has previously been noted that there is a tendency towards a lower number of germ cells in the KS testis already in childhood (2). One study even reported a diminished number of germ cells in fetal life (7), but two other studies reported no such differences (5,6). Here, we evaluated systematically the number of germ cells per tubular area in a total of eight fetal KS testes and 15 age-matched controls. The advantage of using germ cell counts per tubular area instead of per total section area is that the potential difference in amounts and size of tubules per total area is accounted for. Even though the interstitial areas of the fetal KS testis appeared very similar to the normal karyotypic fetal testis, we found a clear tendency towards the lack of differentiation of gonocytes into prespermatogonia with concomitant loss of gonocytes, which indicates that already during fetal life, a significant number of germ cells are lost in the testes from KS fetuses.

In this study, we identified for the first time 211 DETs between the fetal KS testis and age-matched controls. The majority of transcripts were upregulated (153 out of 211), and enriched for transcripts expressed from the X-chromosome. An enrichment of expression of transcripts from the X-chromosome has also been found in previous studies (11,13), and fits very well with the extra X-chromosome in KS. The enrichment and upregulation of the X-chromosomal transcripts indicate that the extra X-chromosome has a direct effect at this developmental time point.

A novel finding of our study is that many of the identified transcripts in the fetal KS testis were ncRNAs. In particular, we found enrichment of many lincRNAs, but only 2 of the 37 lincRNAs were from the X-chromosome, including *XIST*, the best characterized lincRNA. lincRNAs were, apart from *XIST*,

previously considered "junk" RNAs. The majority of lincRNAs have uncharacterized functions, but they are generally believed to have regulatory roles within the cell, in terms of transcription, chromatin modification and protein interaction, among others (20). During the development of the mouse male germline, many lincRNAs are up- and down regulated, indicating an important role of lincRNAs in germ cell development (21). Two recent studies showed that long ncRNAs (lncRNAs) in general are expressed during mouse (22) and human (23) spermatogenesis in a dynamic fashion, underlining the potential importance of lincRNAs in regulating testis function. In the rodent study Wichman *et al.* (22) further showed that knock-out of one of the lncRNAs highly expressed during spermatogenesis, *Tslrn1 (testis-specific long non-coding RNA 1)*, resulted in a decreased sperm production. Still, very limited functional information is available for most of the lincRNA and apart from *XIST*, we were not able to identify any particular lincRNA that potentially could compromise the development of the KS testis. Besides the lincRNAs, the RNA biotype categories sense intronic and antisense, which also are classified as long ncRNAs (lncRNA), were found significantly enriched in the fetal KS testis. Collectively, our data nevertheless indicate that lncRNAs may be involved in the developmental changes identified in the KS testis. The potential role of ncRNAs in germ cell development is highlighted by the importance of a special family of small ncRNAs, the Piwi-interacting RNAs (piRNAs), which are required for maintenance and renewal of germline stem cells (24). Indeed, future studies should elucidate whether these ncRNAs play a role in the development of the testis, and whether their dysregulation in the fetal KS testis is a cause or a consequence of the aberrant germ cell differentiation.

Albeit our study indicates that ncRNAs may be important for the development of the testicular phenotype in KS, protein-
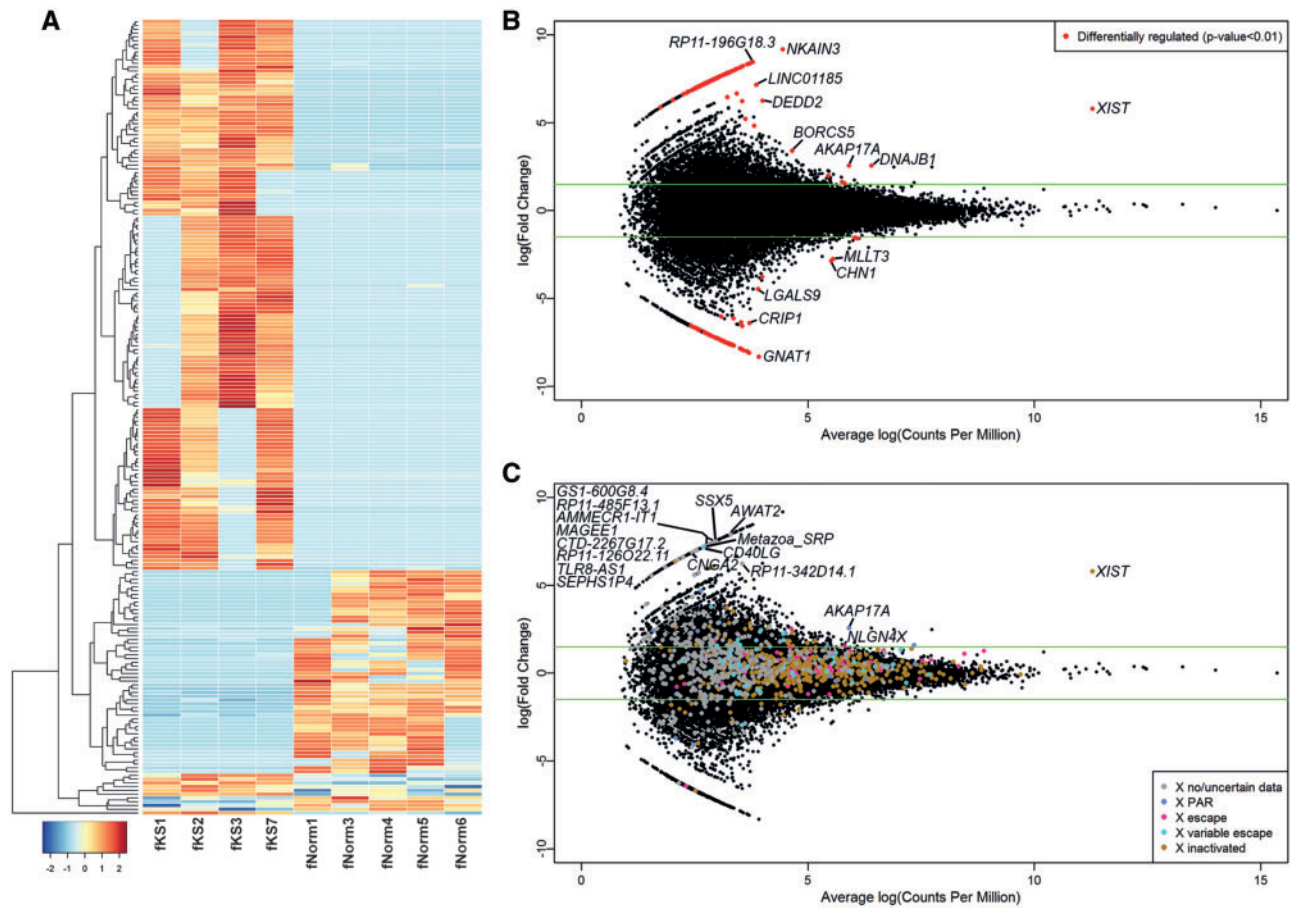
**Figure 3.** Differentially expressed transcripts (DETs) in testes from fetuses with Klinefelter syndrome (KS) vs. controls. (**A**) Heatmap of the DETs (unadjusted *P*-value <0.01) when comparing fetal KS (*n* = 4) with fetal controls (*n* = 5). For sample ages and karyotypes, see Table 1. (**B**) Volcano plot of all expressed transcripts. The DETs are shown with red dots and examples are indicated with transcript names. (**C**) X-chromosomal transcripts expressed in the dataset plotted according to their X-chromosome inactivation status (18). The significantly regulated X-chromosomal transcripts (unadjusted *P*-value <0.01) are shown with transcript names.
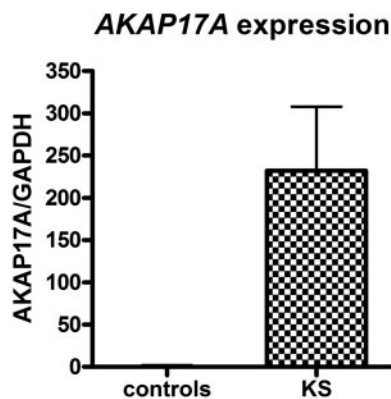


**Figure 4.** Validation of differential expression of *AKAP17A* by quantitative PCR. Expression of *AKAP17A* in two fetal Klinefelter testes (fKS2 and fKS3) and two controls (fNorm3 and fNorm4) as determined by RT-qPCR. The Ct-values of *AKAP17A* were normalized to those of *GAPDH*.

coding transcripts are likely also involved. Among the 75 DETs that were protein-coding, none have previously been described in relation to fetal testis development. Three of the DETs (*VIP*, *RXRA* and *ALX1/CART1*), all of them upregulated in fetal KS samples, have previously been described in relation to postnatal testis function and androgen signaling in animal models.

VIP (vasoactive intestinal peptide) has been shown to be released into the feline testis via the spermatic nerve in the vicinity of Leydig cells (25). In cultured porcine Leydig cells, addition of VIP can increase the production of testosterone and progesterone (26), and in *Vip* knock-out male mice, lower levels of FSH and testosterone were observed. Young males presented with early signs of testicular degeneration and in old mice, testicular aging was delayed (27).

We noted a potential disturbance in germ cell differentiation in the fetal KS testis, and two transcripts previously described in relation to the retinoic acid system, which is essential for peri-pubertal initiation of meiosis in the testis, were identified: RXRA and ALX1/CART1.

RXRA (retinoid X receptor alpha) is involved in regulation of the transition of $A_{Al}$ to $A_1$ spermatogonia in the mouse. RXRA heterodimerizes with the nuclear receptor RARG, and this complex binds to the *Sall4* promoter. In the presence of All-trans retinoic acid (ATRA), the complex is active, so that *Sall4* expression is elevated, which leads to elevation of *Kit* and thereby differentiation of $A_{Al}$ to $A_1$ spermatogonia (28). It is of interest that both SALL4 and KIT are expressed at the protein level in human gonocytes (29–31) and SALL4 in a subset of spermatogonia (31).

ALX1/CART1 has been reported to function as a cytoplasmic retention factor of the nuclear receptor PAR in the mouse Sertoli cell line TM4. Overexpression of CART1 resulted in de-repression
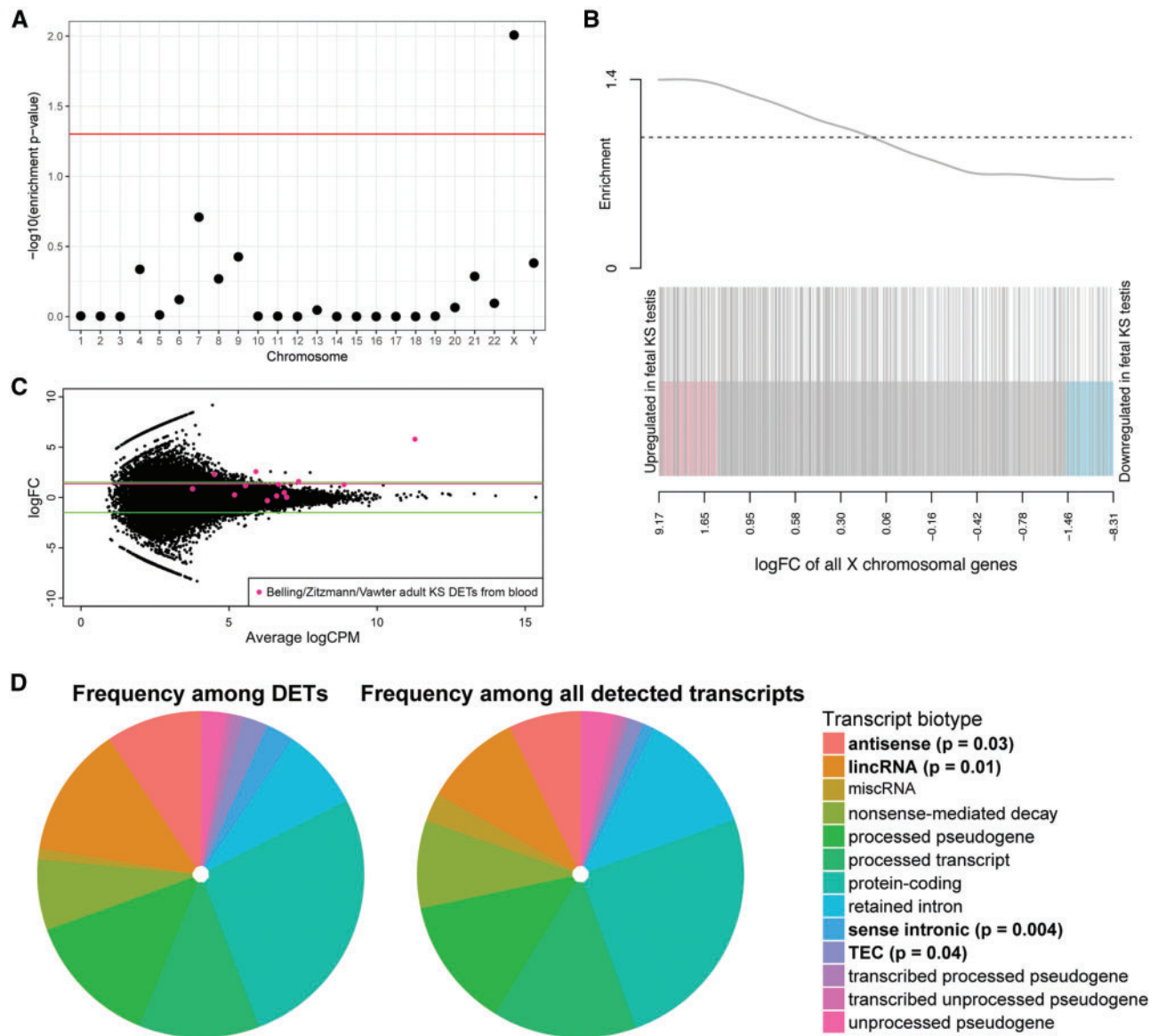
**Figure 5.** Enrichment analysis of the differentially expressed transcripts (DETs) in fetal KS testis. (**A**) Chromosome enrichment analysis. The red line indicates a P-value of less than 0.05. Genes on the X-chromosome were significantly enriched in the dataset. (**B**) Top: Enrichment tendency according to logFC. **Bottom**: Barcode plot showing a tendency of the X-chromosomal transcripts to be upregulated in the dataset. (**C**) Enrichment analysis of 13 DETs identified from at least two of three previous studies performed on RNA from blood (11–13). The pink line indicates the mean upregulation (logFC=1) of this group of transcripts. (**D**) Comparison between transcript biotype distribution among all detected transcripts (right) and the DETs in the KS testis (left). The biotypes marked with bold in the legend were found to be enriched among the DETs. P-values originate from a hypergeometric test. For a description of each RNA biotype, see Supplementary Material, Table S3. FC: fold change, TEC: to be experimentally confirmed.

of PAR in the absence of retinoic acid, thereby leading to a basal transcription of retinoic acid-target genes in the absence of retinoic acid (32). It cannot be assumed that the effect of CART1 upregulation is similar to the addition of retinoic acid, but one could speculate that CART1 upregulation could lead to similar effects as retinoic acid, but to a lower extent. The effects of retinoic acid on early fetal testis development have previously been investigated in a study using an *ex vivo* hanging drop organ culture system of first trimester testis samples (33). In that study, addition of retinoic acid did not lead to initiation of meiosis as in the fetal ovary, but instead a significant decrease in the number of OCT3/4-positive gonocytes as well as less proliferation of these was

observed (33). Based on our data that indicate failure of gonocyte differentiation into pre-spermatogonia, it could be speculated that CART1 is one of the genes involved in this process, but further studies are needed to corroborate this hypothesis.

In addition, *DEDD2* has been shown to be a nucleolus-targeted pro-apoptotic protein depending on caspase-8 and -10-activity in various cell culture systems (34). Since we found significantly lower numbers of pre-spermatogonia in fetal KS testes, it is possible that gonocytes undergo apoptosis instead of differentiating, indicating the upregulation of *DEDD2* in the fetal KS samples as a potential mechanism for induction of apoptosis. To examine whether the germ cells do undergo apoptosis,

we did IHC with cleaved poly ADP ribose polymerase (cPARP) which is a marker of apoptosis. We saw only a few positive cells in all samples, and no systematical differences between the samples (data not shown). However, because testicular samples represent static pictures in the development of the fetal testis, it cannot be completely ruled out that germ cells do undergo apoptosis in the fetal KS testis.

We were able to validate a high overexpression of the PAR1-encoded transcript *AKAP17A* in fetal KS by RT-qPCR. AKAP17A (A-kinase anchor protein 17A), also known as XE7, is involved in regulation of pre-mRNA splicing in a Protein Kinase A-dependent manner (35). According to the GTex portal (36), *AKAP17A* shows ubiquitous expression in adult tissues, but not at the protein level according to GeneCards (37). At the protein level, AKAP17A is found, among other places, in the blood, ovaries and testis. *AKAP17A* has previously been identified as being upregulated in the blood of KS patients (11,13), thereby strengthening the evidence of its potential role in KS. Moreover, AKAP17A has been related to chronic tic disorder and tics are sometimes observed among men with KS (38,39).

We have to mention that the transcriptome analysis on archival FFPE testis tissue was technically very challenging. The sample availability was very limited, and in all cases, the samples were fixed material where the gonadal regions had to be isolated before RNA extraction. Besides challenges with sparse amount of material, the quality of the extracted RNA was further compromised, because of formalin-fixation and long-term storage. After thorough quality control, we had to exclude a total of five samples, decreasing the series to 4 KS and five controls representing the best possible data from these challenging and precious samples. Because we used RNA from whole gonadal regions, we cannot know whether the observed differences are due to an actual differential regulation of those particular transcripts or a systematic difference in cell types between the fetal KS and controls. Moreover, the limited sample availability did not allow us to completely match ages of the fetal samples, which imply that samples are at different development stages. However, we know that at least the human gonocyte differentiation to spermatogonia is asynchronous and takes a much longer time than in rodents (40,41). We tried to account for the slight age differences by doing a sanity check for expression of known Sertoli cell, peritubular cell and Leydig cell markers, and found no systematic differences between the groups, although we saw a tendency for one sample, fNorm5, to have a low expression of all markers examined. We evaluated the amount of germ cells and saw a difference between the eldest fetal KS and the eldest controls, but other than that the number of germ cells was similar between earlier/younger KS and controls. Also, because we measured the total area of tubules in each sample, we could see that the proportion of tubules, and thereby the proportion of the interstitial area per total area did not differ considerably between the two groups. But still, one has to consider the possibility that the differential expression could be due to subtle differences in cellularity. Nevertheless, despite all challenges, the fetal material from KS individuals is unique and the study provided novel insights concerning the early stages of testicular failure in KS.

In conclusion, this is the first study of the transcriptome and germ cell maturation pattern in fetal KS testes and indicates that loss of germ cells, which is characteristic for the adult KS testicular phenotype, is initiated already in fetal life by failure of the gonocyte differentiation into pre-spermatogonia. Elucidation of the mechanisms requires further studies, but our dataset identifies several candidate genes/pathways, including a possible role of aberrant expression of lncRNAs.

## Materials and Methods

### Tissue samples

The use of tissue was approved by a regional medical and research ethics committee (H-2-2014-103) and the Danish Data Protection Agency (2012–58-0004, local no. 30–1482, I-Suite 03696). Fetal testes were collected between 1993 and 2012 at autopsy of induced abortions due to fetal KS or from spontaneous abortions of normally developed male fetuses. Gestational age varied between 13 and 22 weeks. The samples were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. The blocks were stored at $-20\,°C$ until sectioning.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed essentially as described before (42). Antibody details and unmasking buffers are shown in Supplementary Material, Table S4. Staining with anti-MAGE-A4 and anti-OCT3/4 antibodies was performed on serial sections of testes from eight fetuses with KS and 15 controls (Table 1). In brief, paraffin-embedded sections were deparaffinized and rehydrated. Antigen retrieval was accomplished by microwaving the sections for 15 min in unmasking buffer. Then sections were incubated with 2% non-immune goat serum (Zymed Histostain Kit, Life Technologies, CA, USA) to minimize cross-reactivity. The primary antibody was added and incubated for 16 h at $+4\,°C$, and then the sections were incubated with biotinylated goat anti-mouse IgG, before a peroxidase-conjugated streptavidin complex was used as a tertiary layer (Zymed Histostain Kit). Visualization was performed with amino ethyl carbasole (Zymed Histostain Kit).

### Quantification of positive stained cells per tubular area

Adobe Photoshop CC 2014 (Adobe Systems Inc, CA, USA) was used to quantify the amount of tubular cells stained positive for either OCT3/4 (gonocytes) or MAGE-A4 (pre-spermatogonia). All positive cells, whether they were in longitudinally or transversely cut tubules, were counted using the count tool, and the total tubular and total area in $mm^2$ were measured with the lasso tool. GraphPad Prism version 4 (GraphPad Software Inc, CA, USA) was used to plot the data.

### Tissue sectioning and microdissection

FFPE tissue was sectioned on a microtome at a thickness of 8 μm, and placed on RNase-free MMI MembraneSlides (Molecular Machines & Industries, Eching, Germany). Before microdissection, membranes were incubated at $56\,°C$ for 1 h, and then deparaffinized with xylene, followed by rehydration, staining with Mayer's hematoxylin, and dehydration. All procedures were performed using baked glassware and DEPC water (Sigma Chemical Company, MO, USA). Serial sections of each tissue were collected before and after the collection on membranes and stored at -20 °C for subsequent histological analysis. In order to remove unwanted tissues such as epididymis, large blood vessels and connective tissue, the testes were microdissected on an MMI CellCut LMD system (Molecular Machines & Industries) (Supplementary Material, Fig. S3).

## RNA extraction

Total RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific, MS, USA) according to the manufacturer's recommendations. To assess the quantity and quality of the samples, the Qubit RNA HS Assay Kit (Thermo Fisher Scientific) and the Agilent RNA 6000 Pico Kit (Agilent Technologies, CA, USA), respectively, were used.

## Library preparation

The Ovation Human FFPE RNA-Seq Multiplex Systems (NuGEN, Leek, Netherlands) were used for library preparation from 250 ng of total RNA according to the manufacturer's recommendations with the following modifications: The cDNA samples were fragmented to yield sizes of approximately 150 bp using the Covaris E210 sonicator (Covaris Ltd., Brighton, UK). For the final library amplification, 20 cycles of amplification were used. An extra DNA purification was performed with a 1.4 times overload of Agencourt AMPure XP beads (Beckman Coulter, IN, USA) to get rid of adaptors and primers. Samples were pooled with equal amount of each. We performed 125 nt paired-end read sequencing on the Illumina HiSeq 2500 sequencing platform (Illumina, CA, USA).

## Analysis of RNA-sequencing data

Reads were demultiplexed using a custom Perl script and adapter sequences trimmed using AdaptorRemoval v1.5 (43). Reads were mapped to the human reference genome (GRCh38) in which the pseudoautosomal regions on the Y-chromosome (chrY: 10001–2781479, chrY: 56887903–57217415) were masked using STAR v. 2.5.2b (44). Read counts were summarized using HTseq v. 0.6.0 (45).

Data were analysed in the R software version 3.3.2 (46). Briefly, the count data were loaded into the limma/edgeR package (47,48) and heatmaps were generated with the package gplots (49) using the heatmap.2 function. Fold changes were calculated as the mean expression values and then log2-transformed. Based on this, three KS (at Gw15, Gw19, and Gw21) and two controls were excluded (at Gw14 and Gw22) (Fig. 2 and Supplementary Material, Fig. S1). To avoid accidental findings, only transcripts with at least 1 count per million in at least three samples were included, which left a total of 27, 610 transcripts.

The R package limma (50) was used to identify DETs as well as enrichment analysis (mroast and camera functions). XCI status was acquired from Balaton *et al.* (18) and GENCODE transcript biotypes were obtained from Ensembl using the biomaRt package in R (51,52). We tested for enrichment of transcript biotypes the phyper function in R.

Data have been deposited in the GEO public database with the accession number GSE103613.

## RT-QPCR validation

RNA was amplified using the amplification reagents from the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (Takara Bio Europe AB, Saint-Germain-en-Laye, France) according to the manufacturer's recommendations with 11 cycles of cDNA amplification. cDNA was purified with a one time overload of Agencourt AMPure XP beads (Beckman Coulter). qPCR was performed with Taqman® Fast Advanced Master Mix (Thermo Fisher Scientific) using the Gene Expression Assays: AKAP17A Hs00946622_m1 and GAPDH Hs004420632_g1 (Thermo Fisher Scientific). PCR conditions were: 50 °C for 2 min, 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 62 °C for 20 s. The plate was run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The Ct-values for *AKAP17A* were normalized to the ones of *GAPDH*. Data were plotted using GraphPad Prism.

## Supplementary Material

Supplementary Material is available at *HMG* online.

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