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Developmental Changes in Human Fetal Testicular Cell Numbers and Messenger Ribonucleic Acid Levels during the Second Trimester

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Context: Normal fetal testis development is essential for masculinization and subsequent adult fertility. The second trimester is a critical period of human testicular development and masculinization, but there is a paucity of reliable developmental data.

Objective: The objective of the study was to analyze second-trimester human testicular morphology and function.

Design: This was an observational study of second-trimester testis development.

Setting: The study was conducted at the Universities of Glasgow and Aberdeen.

Patients/Participants: Testes were collected from 57 morphologically normal fetuses of women undergoing elective termination of normally progressing pregnancies (11–19 wk gestation).

Main Outcome Measure(s): Testicular morphology, cell numbers, and quantitative expression of 22 key testicular genes were determined.

Results: Sertoli cell and germ cell number increased exponentially throughout the second trimester. Leydig cell number initially increased exponentially but slowed toward 19 wk. Transcripts encoding Sertoli (*KITL*, *FGF9*, *SOX9*, *FSHR*, *WT1*) and germ (*CKIT*, *TFAP2C*) cell-specific products increased per testis through the second trimester, but expression per cell was static apart from *TFAP2C*, which declined. Leydig cell transcripts (*HSD17B3*, *CYP11A1*, *PTC1*, *CYP17*, *LHR*, *INSL3*) also remained static per cell. Testicular expression of adrenal transcripts *MC2R*, *CYP11B1*, and *CYP21* was detectable but unchanged. Expression of other transcripts known or postulated to be involved in testicular development (*GATA4*, *GATA6*, *CXORF6*, *WNT2B*, *WNT4*, *WNT5A*) increased significantly per testis during the second trimester.

Conclusions: The second trimester is essential for the establishment of Sertoli and germ cell numbers. Sertoli and Leydig cells are active throughout the period, but there is no evidence of changing transcript levels. **(*J Clin Endocrinol Metab* 92: 4792–4801, 2007)**

DIFFERENTIATION AND DEVELOPMENT of the male reproductive tract is critically dependent on normal testicular development. Androgen, IGF 3 (INSL3) and anti-Mullerian hormone, secreted by the Leydig cells and Sertoli cells, ensure masculinization of the internal ducts and normal development of the male phenotype, while the maintenance and differentiation of the male primordial germ cells depend on the Sertoli cells. In the human, testicular differentiation begins at about 6 wk after conception with the gradual development of testicular cords containing Sertoli cells and primordial germ cells (1). The Sertoli cells rapidly begin to produce anti-Mullerian hormone, driving the process of Mullerian duct regression (2), which is completed before midgestation (3). Around wk 8 Leydig cells begin to differentiate outside the cords (4) with increasing levels of androgen detectable in the gonad thereafter (5). Wolffian duct differentiation occurs between 9 and 13 wk (reviewed in Ref. 6), while masculinization of the external genitalia occurs between 8 and 12 wk gestation (reviewed in Ref. 7). Furthermore, it is during the 10–14th wk gestation that testis descent begins, and this is initially stimulated by INSL3 (8, 9). Despite the clear importance of the second trimester for testicular development and fetal masculinization, reliable measures of developmental changes in testicular cell numbers, using modern techniques, are lacking. Similarly, there is a lack of detailed study of developmental changes in cell function during this period, despite success in establishing normal developmental patterns in other species (*e.g.* Refs. 10–13). Consequently, in this study we have measured changes in testicular cell number in the human fetus during the second trimester and have quantified transcript levels of key mRNA species that encode proteins likely to be involved in fetal testis development and function.

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Abbreviations: hCG, Human chorionic gonadotropin; INSL3, IGF 3; MCR, melanocortin receptor.

Subjects and Methods

Subjects

The collection of fetuses complied with the Polkinghorne Committee recommendations and was approved by the National Health Service Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective, medical terminations of pregnancy were recruited with written, informed consent, independently by nurses at Aberdeen Pregnancy Counseling Service, with no involvement of the authors. Recruitment involved no change in patient treatment or care, and women were

able to withdraw from the study at any point. Only normally progressing pregnancies at ultrasound scan, from women older than 16 yr of age with a good grasp of English and between 11 and 19 wk gestation were collected. Induced abortions were performed under the terms of the 1967 Abortion Act (United Kingdom). After appropriate counseling, each woman received a single tablet of mifepristone 200 mg, swallowed under supervision. Women were admitted to the gynecology ward 36–48 h later, and four tablets (a total of 800 μg) of misoprostol were inserted into the posterior vaginal fornix. Three hours after the first dose of misoprostol, 400-μg doses were administered orally at 3-h intervals, to a maximum of four doses. Specimens for this study were collected only if products were delivered within 9 h of prostaglandin treatment starting. The precise time at which fetal death occurred for any one sample is not known, but the maximum interval between fetal death and specimen collection would be less than 6 h. It has been shown previously that little or no change in specific mRNA quality or quantity will occur within this time span (14). Any fetal abnormalities observed in the laboratory resulted in the return of the fetus to the ward without tissue collection. Fetuses were transported to the laboratory within 30 min of delivery, weighed, crown-rump length recorded, and sexed. An *ex vivo* blood sample was then collected by cardiac puncture when possible and the plasma stored at −20 C. The gonads were dissected, weighed, and one was snap frozen in liquid nitrogen and stored at −80 C, whereas the other was fixed for 5.5 h in Bouins and then stored in 70% ethanol. In this study the left testis was used for stereology and the right testis for measurement of mRNA levels. In total, 57 fetuses were used for this study.

Stereology and histology

For stereology, testes were embedded in Technovit 7100 resin, cut into 20-μm sections, and stained with Harris' hematoxylin. For histology 5-μm sections were cut and stained with hematoxylin and eosin. The total testis volume was estimated using the Cavalieri principle (15), and the slides used to estimate the number of cells were also used to estimate testis volume. The optical dissector technique (16) was used to count the number of Leydig, Sertoli, and germ cells in each testis. Sertoli and germ cells were identified by their distinctive nuclei and position within the tubule (17, 18), whereas the Leydig cells were identified by their position within the interstitial tissue and by their round nuclei and prominent nucleoli (17, 18). Total germ cell number was measured without categorization as gonocytes, intermediate cells or prespermatogonia (19–21). The numerical density of each cell type was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA). Cross-sectional tubule diameters were measured in six fields of view from two sections of each fetal testis using an Olympus BX41 at x20 with a ProgRes C5 digital camera and ProgRes CapturePro software (Jentopik Laser Optic Systeme GmbH, Jena, Germany).

Measurement of mRNA levels

For quantification of the content of specific mRNA species in testes during development, a real-time PCR approach was used after reverse transcription of the isolated RNA. To allow specific mRNA levels to be expressed per testis and to control for the efficiency of RNA extraction, RNA degradation, and the reverse transcription step, an external standard was used (22, 23). The external standard was luciferase mRNA (Promega UK, Southampton, UK), and 5 ng were added to each testis at the start of the RNA extraction procedure. Testis RNA was extracted using TRIzol (Life Technologies, Paisley, UK), and the RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies) as described previously (24). The real-time PCR approach used the SYBR green method in a 96-well plate format using a Stratagene MX3000 cycler. Reactions contained 5 μl 2 × SYBR mastermix (Stratagene, Amsterdam, The Netherlands), primer (100 nM), and template in a total volume of 10 μl. At the end of the amplification phase, a melting curve analysis was carried out on the products formed. For mRNA transcripts that are known to be expressed in one cell type only, data have been expressed per testis (*i.e.* relative to luciferase) and per cell (testis expression divided by cell number). All primers were designed by Primer Express 2.0 (Applied Biosystems, Warrington, UK) using parameters previously described (25). The primers used are described in supplemental Table 1 (published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). The melanocortin-2 receptor (*MC2R*) amplicon was sequenced directly using big dye terminator cycle sequencing kits (Applied Biosystems).

Testosterone assay

Testosterone was determined using a single DELFIA kit (PerkinElmer Life Sciences, Cambridge, UK) with a detection limit of 0.3 nmol/liter and intra- and interassay coefficients of variation of 8.6 and 3.2%, respectively. Many of the samples were hemolyzed, risking interference with the assay. Consequently, all 34 fetal plasma samples available were processed through 250 μl C18 columns (Self Pack POROS 10 R2; Applied Biosystems) with a binding capacity of 10 μg/μl. Columns were washed with acetonitrile and water, and aliquots (50 μl) of either normal adult male plasma or fetal plasma were then loaded onto the columns and washed with milliQ water. Bound steroid hormone was eluted with 2 ml methanol and evaporated at room temperature. Dried samples were reconstituted with 50 μl of DELFIA testosterone assay buffer (PerkinElmer Life Sciences) and 25-μl aliquots used for assay. The repeated recovery of testosterone was 122 ± 9.7%, representing a slight overestimate of titers in the fetal samples.

Statistical analysis

Analyses were performed using JMP (version 5.1) software (Thomas Learning, London, UK). Normality of data distribution was tested with the Shapiro-Wilk test, and nonnormally distributed data were log transformed before analysis. In all cases this transformation led to normal distribution of the data. Single-factor ANOVA was used to test the effects of gestational age both as weeks and as grouped weeks (7–9, 11–13, 14–16, 17–19) on morphological, biochemical, and gene expression data. Relationships between variables were explored by linear correlation using Pearson's product moment correlation coefficient. Curves were fitted by nonlinear regression using a second-order polynomial or by linear regression after log transformation.

Results

Testis morphology

The morphology of the testis was consistent with that described previously for the human fetal testis (17, 26). On wk 11 the testes were clearly separated into tubular and interstitial compartments, and the cell types of interest (Leydig, Sertoli, and germ cells) were already distinctive. At 19 wk there was no clear change in overall morphology, although the tubules grew in cross-sectional diameter from 103 ± 6 μm at 11–13 wk to 137 ± 8 μm at 17–19 wk ($P = 0.010$, n = 20). There was no difference in weight between left and right testis (all samples; 14.7 *vs.* 15.1 mg, SD 10).

Changes in cell number during the second trimester

Testis volume increased exponentially during the period of the study (Fig. 1). The mean value was 12.2 mm^3, which compares with 19 mm^3 in an earlier study for an older cohort (13–25 wk) (27). Leydig cell number increased during the second trimester and the best fit of the data suggest that there was an exponential rise in the first part of the second trimester, which slowed down after about 15 wk. Sertoli cell number increased exponentially throughout the second trimester (Fig. 1). Combining this data with that previously obtained by another group from the first trimester (26) shows that the exponential increase during the second trimester is a continuation of the rate set in the first trimester. From this data the apparent doubling time for Sertoli cells was 14 d.

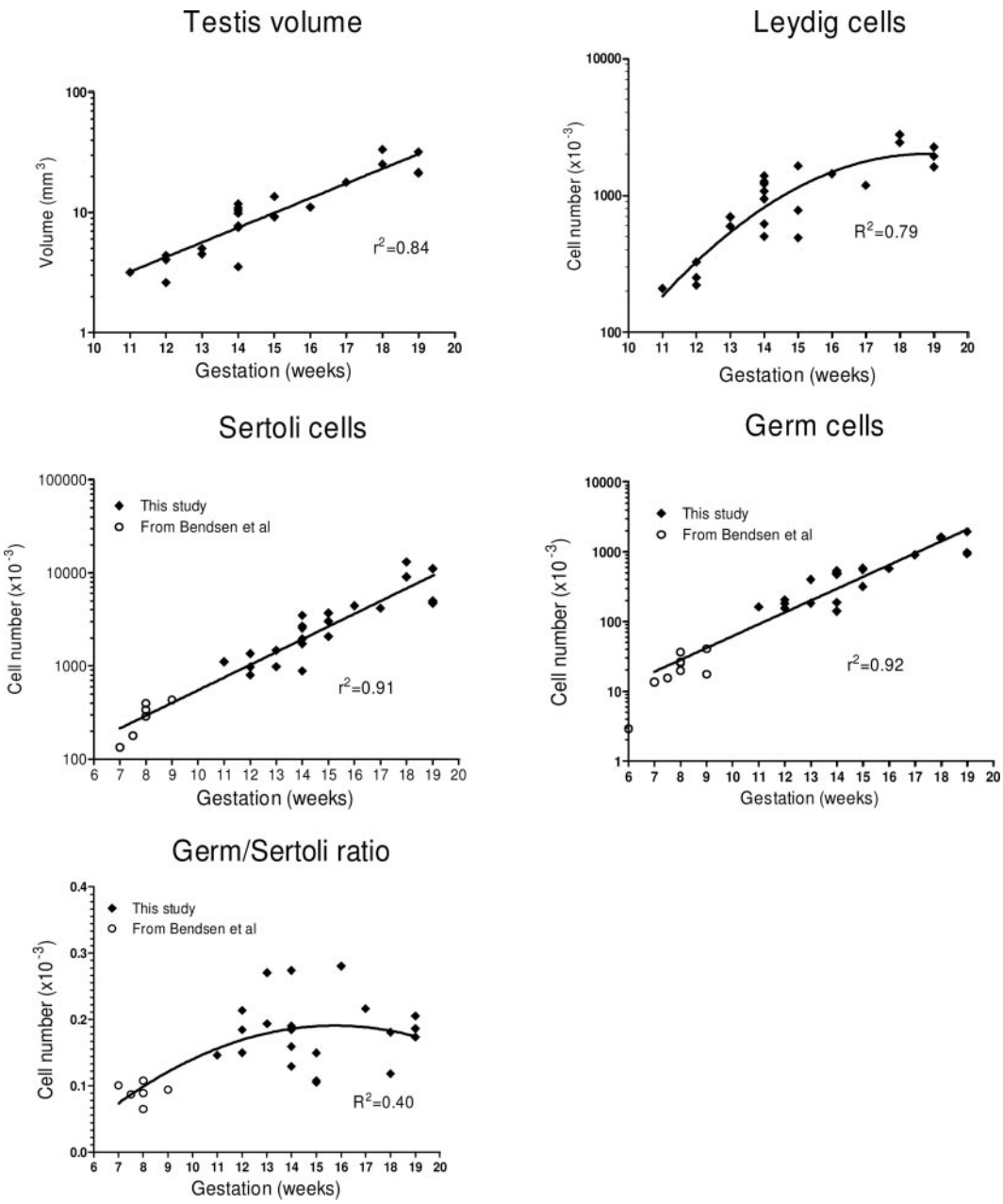

FIG. 1. Changes in human fetal testicular volume, testicular cell numbers, and germ cell to Sertoli cell ratio during the second trimester. Data shown were generated during this study (♦) (n = 22) or are taken from the reported study of Bendsen *et al.* (26) (○). Curves were fitted by linear regression following log transformation apart from the data for Leydig cells and the ratio of germ cells to Sertoli cells, which were fitted by nonlinear regression using a second-order polynomial. Individual data points are shown. There was a significant increase in testicular volume and cell number with weeks of gestation ($P < 0.05$). The difference in germ cell ratio between the data reported by Bendsen *et al.* (26) and that generated by this study was also significant ($P < 0.05$).

Germ cell number also increased exponentially from about wk 7 to wk 19 with an apparent doubling time of 12 d. The ratio of germ cells to Sertoli cells in the 7- to 9-wk period was about 0.1 (data from Ref. 26), and this doubled to about 0.2 after 12 wk ($P < 0.05$) (Fig. 1).

Fetal testosterone

Fetal plasma testosterone concentrations were variable between individual fetuses of the same age (Fig. 2A), but concentrations in the early trimester (11–13 wk) were significantly higher ($P < 0.05$) than those in the late trimester (17–19 wk), confirming a significant fall in testosterone across the period.

Changes in gene expression during the second trimester

Leydig. Figure 2B shows expression of known and putative Leydig cell genes during human testis development. Levels of *HSD17B3*, *CYP11A1*, and *PTC1* mRNA increased significantly

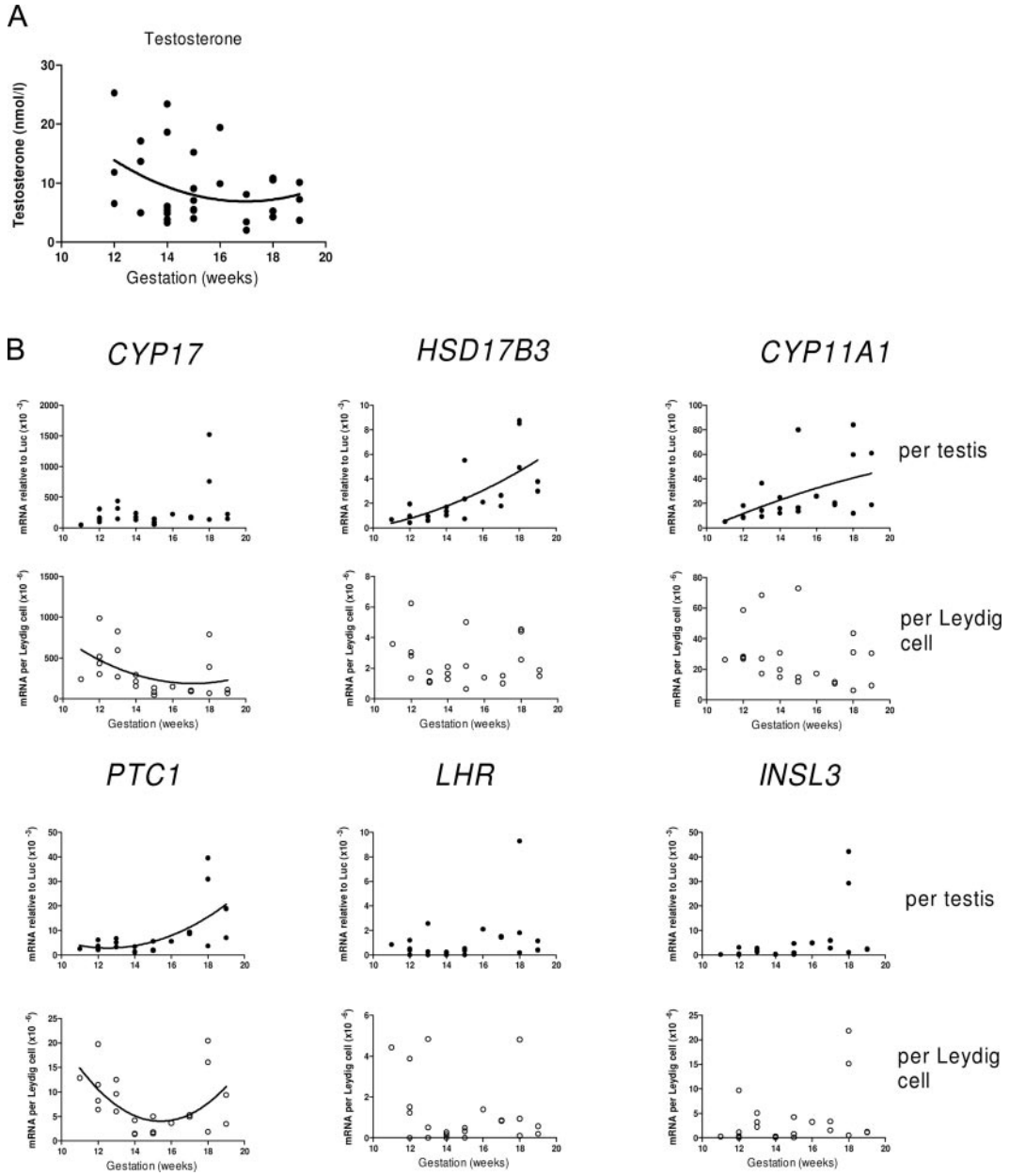

FIG. 2. A, Changes in fetal plasma testosterone levels during the second trimester (n = 34). Individual data points are shown at each age, and the curve was fitted by nonlinear regression using a second-order polynomial. B, Changes in levels of mRNA encoding Leydig cell-specific transcripts in the human fetal testis during the second trimester (n = 22). RNA was extracted from testes at different ages and cDNA prepared as described in *Subjects and Methods*. Real-time PCR was used to measure cDNA levels relative to an external standard (luciferase) added during RNA extraction, and results are shown as expression per testis (*upper graph of each gene*) or normalized to the number of Leydig cells present at each age (*lower graph of each gene*). Curves were fitted by nonlinear regression using a second-order polynomial. No curve is shown where R^2 is less than 0.2. Individual data points are shown. Levels of *HSD17B3*, *CYP11A1*, and *PTC1* mRNA increased significantly ($P < 0.05$) per testis during the second trimester. There was no developmental change in levels of any mRNA transcript per Leydig cell.

per testis through the second trimester, but there were no significant changes in *CYP17A1*, *LHR*, or *INSL3* levels. None of the transcripts measured showed any significant change in expression per Leydig cell over the same period (Fig. 2B).

The adrenal proteins MC2R, CYP21A1, and CYP11B1 are expressed in the fetal mouse Leydig cell (27, 58, 59), and expression was detectable in the human fetal testis, although there was no significant change in expression with age (Fig. 3).

Sertoli cells. Developmental changes in selected Sertoli cell genes are shown in Fig. 4. Expression levels of all mRNA species tested (*KITL*, *FGF9*, *SOX9*, *FSHR*, *WT1*) increased significantly per testis during the second trimester. When normalized per

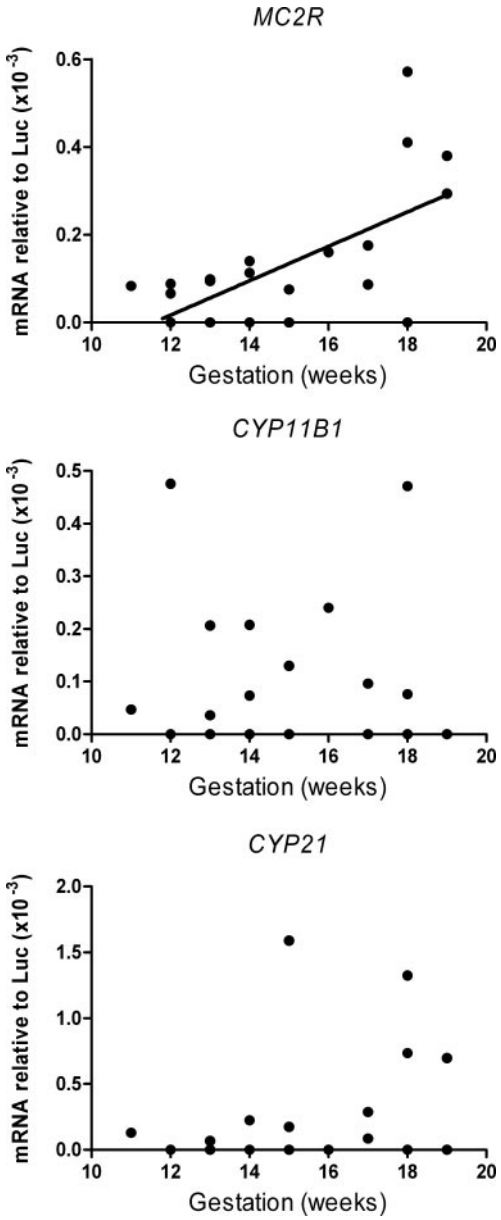

FIG. 3. Changes in levels of fetal testicular mRNA encoding transcripts associated with adrenal steroidogenesis during the second trimester (n = 22). RNA was extracted from testes at different ages and cDNA prepared as described in *Subjects and Methods*. Real-time PCR was used to measure cDNA levels relative to an external standard (luciferase) added during RNA extraction. Curves were fitted by nonlinear regression using a second-order polynomial. No curve is shown where R^2 is less than 0.2. Individual data points are shown.

Sertoli cell, however, there were no significant changes in mRNA levels of any of the transcripts measured (Fig. 4).

Germ cells. Levels of mRNA encoding two germ cell-specific products (CKIT, TFAP2C) increased significantly per testis during the second trimester (Fig. 5). When normalized per germ cell number, however, *CKIT* levels did not change and *TFAP2C* actually declined over the same period ($P < 0.05$).

Transcription factors and developmental signaling genes. In addition to genes known to be selectively expressed in one cell type, we examined expression of a number of genes that either show a wider expression pattern or whose expression pattern is unknown (Fig. 6). The transcription factors GATA4 and GATA6 are expressed in both Sertoli and Leydig cells (28–30), whereas CXORF6 is a causative gene for hypospadias and is also expressed in both Sertoli and Leydig cells (30). Figure 6A shows that expression of each of these mRNA species increased significantly during the second trimester. The WNT proteins are secreted signaling molecules and expression levels of *WNT2B*, *WNT4*, and *WNT5A* mRNA increased significantly through the second trimester.

Discussion

The second trimester is a critical period of male reproductive development during which internal and external genitalia are established, and there is significant development of both somatic and germinal components of the testis. Disturbance of normal testicular development during the second trimester will affect subsequent adult reproductive health and efficiency. In this study we have, therefore, we characterized the normal structural and functional development of the human fetal testis during this period.

Development of adequate numbers of fetal Sertoli and germ cells is critical in establishing the adult population of each cell type. Sertoli cell number at birth in the human is around 260×10^6, which increases to about 3700×10^6 in adulthood (31). In our study Sertoli cell number doubled every 2 wk from wk 7 gestation forming approximately 13×10^6 cells by wk 19 (26 and this study). Reaching a normal birth number would require four more rounds of doubling in the remaining 19 wk. A reduced rate of increase in Sertoli cell number during the third trimester would fit with the slow rate after birth. It is highly likely that the most rapid growth in Sertoli cell number occurs during the latter half of the first trimester and the second trimester and that these periods are crucial, therefore, for the establishment of normal Sertoli cell numbers. The rate of apoptosis in Sertoli cells is low and constant across gestation (32), indicating that it is the rate of proliferation, rather than a balance between proliferation and apoptosis, which determines Sertoli cell number during this period.

From earlier studies it is clear that, in contrast to rodents, human fetal germ cells are not homogeneous, with three separate populations (gonocytes, intermediate, and prespermatogonia) (19, 21). Gonocytes predominate in the first trimester, gonocytes and intermediate cells predominate in the second trimester (although all three cell types are present), and by the third trimester, prespermatogonia become most abundant (20, 21). We did not differentiate among the three germ cell types, and our data represent the sum of the germ cell populations at each fetal age. A previous study has reported that germ cell number in the third trimester is 3.6×10^6 (33), which agrees with germ cell numbers measured at the end of the second trimester in this study. This also suggests that as the fetus moves into the third trimester germ cell numbers become more static. While germ and Sertoli cell numbers showed the same pattern of development through the first and second trimesters, the germ cell to Sertoli cell ratio increased sig-

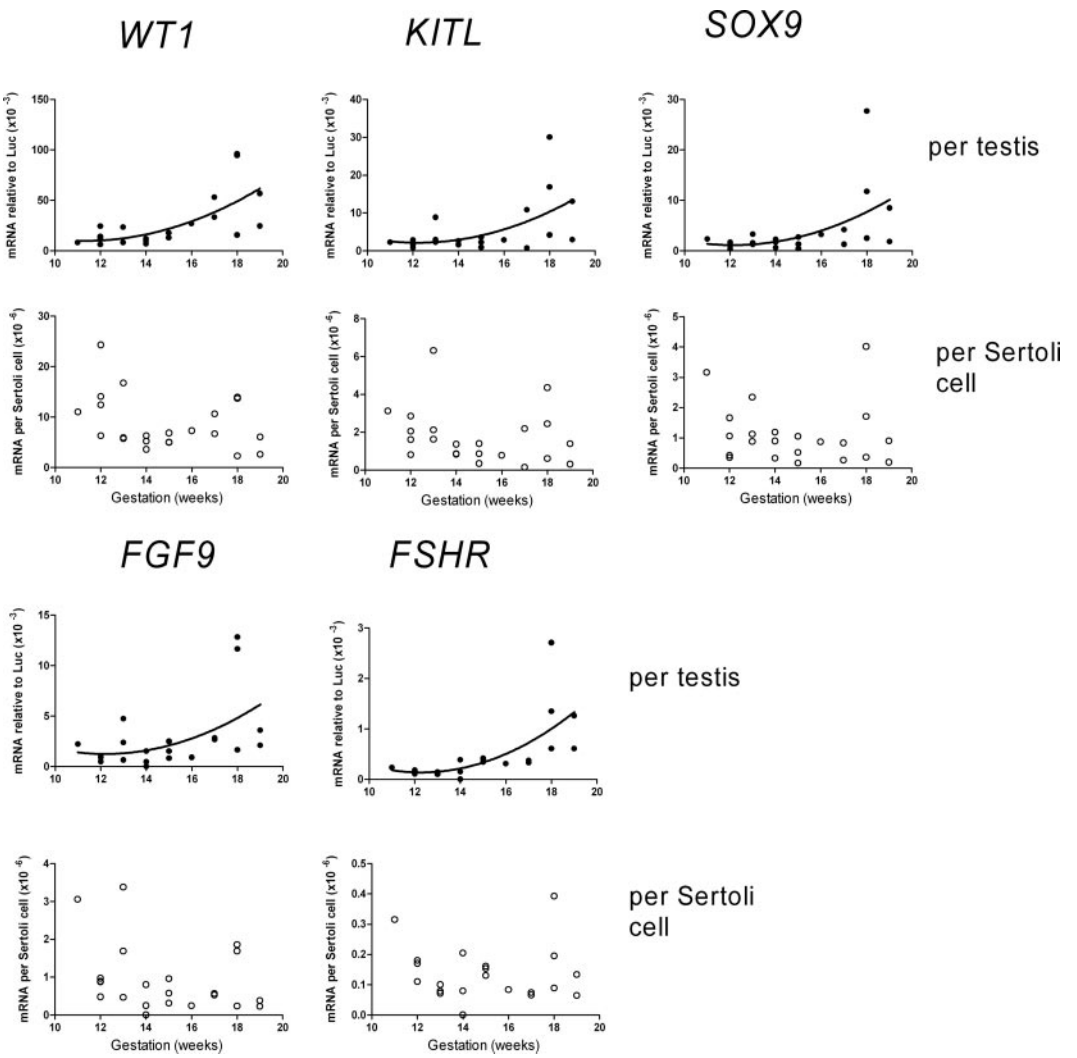

FIG. 4. Changes in levels of mRNA encoding Sertoli cell-specific transcripts in the human fetal testis during the second trimester (n = 22). RNA was extracted from testes at different ages and cDNA prepared as described in *Subjects and Methods*. Real-time PCR was used to measure cDNA levels relative to an external standard (luciferase) added during RNA extraction, and results are shown as expression per testis (*upper graph of each gene*) or normalized to the number of Sertoli cells present at each age (*lower graph of each gene*). Curves were fitted by nonlinear regression using a second-order polynomial. No curve is shown where R^2 is less than 0.2. Individual data points are shown. Levels of all mRNA species measured increased significantly ($P < 0.05$) per testis during the second trimester. There was no developmental change in levels of any mRNA transcript per Sertoli cell.

nificantly over the period. Data from the first trimester come from a different study and a different laboratory (26), but the techniques used in both studies were very similar, and absolute cell numbers from the two studies fit well to the same exponential curve. This provides some confidence that the change in cell ratios is reliable and suggests that maturation of the Sertoli cells at the start of the second trimester allows them to support more germ cells. Continuous Sertoli and germ cell proliferation through the second trimester is consistent with a previous study showing that expression of proliferating cell nuclear antigen is constant, as a percentage of cells, in the human fetal tubule during the second trimester (34).

Leydig cell numbers increased exponentially during the initial part of the second trimester, but by the end of the second trimester, numbers were static. Several studies have reported that absolute numbers of fetal Leydig cells start to decrease at the end of the second trimester through regression (dedifferentiation) or degeneration (17, 35–38), consistent with static numbers of fetal Leydig cells toward the end of the second trimester. This also agrees with falling interstitial proliferating cell nuclear antigen expression toward the end of the second trimester (34).

The genes chosen to test Sertoli cell function are expressed exclusively in the Sertoli cell, and most of the encoded proteins function primarily during testicular differentiation and development (39–41). Previous expression/function studies of these genes have used the mouse as a model system, and there are no direct comparator studies available in the human, the exception being the FSH receptor, which has been shown by hormone-binding studies to be present early in human fetal testis develop-

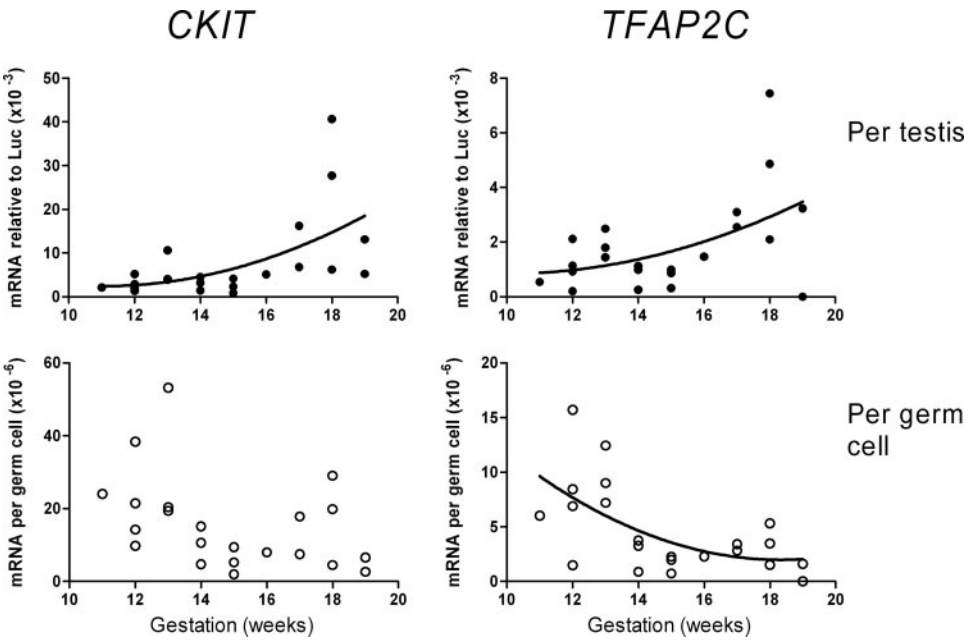

FIG. 5. Changes in levels of mRNA encoding germ cell-specific transcripts in the human fetal testis during the second trimester (n = 22). RNA was extracted from testes at different ages and cDNA prepared as described in *Subjects and Methods*. Real-time PCR was used to measure cDNA levels relative to an external standard (luciferase) added during RNA extraction, and results are shown as expression per testis (*upper graph of each gene*) or normalized to the number of germ cells present at each age (*lower graph of each gene*). Curves were fitted by nonlinear regression using a second-order polynomial. No curve is shown where R^2 is less than 0.2. Individual data points are shown. Levels of all mRNA species measured increased significantly ($P < 0.05$) per testis during the second trimester. Levels of *CKIT* mRNA per germ cell did not change during development, whereas there was a significant decline in *TFAP2C* per germ cell.

ment (42). Expression levels of the five mRNA species measured increased significantly per testis across the second trimester. Over the same period, Sertoli cell number increased around 10-fold, and, expressed per Sertoli cell, there was no significant change in mRNA levels. Thus, it appears likely that there are no marked changes in Sertoli cell activity during the second trimester. In the mouse, no marked changes in Sertoli cell activity during fetal development have been reported once testicular differentiation has been established (43). Significant changes in mouse Sertoli cell mRNA expression are not seen in the mouse until around postnatal d 10, when Sertoli cell numbers are close to adult levels (18, 23). Data from the human are therefore consistent with a relative quiescence in Sertoli cell activity during the proliferative phase of development.

Levels of mRNA encoding germ cell-specific proteins also increased significantly per testis through the second trimester. When expressed per germ cell, however, there was either no change in expression or a slight decline with development. Both germ cell markers measured have been reported to show increasing incidence of expression within the germ cell population as the second trimester progresses (20). It is likely therefore that increased mRNA levels per testis reflect increasing germ cell numbers, whereas static or decreasing levels per germ cell reflect dynamic changes in the different germ cell populations as they develops toward a spermatogonial phenotype (19–21).

Changes in the expression levels of mRNA species that code for Leydig cell proteins were variable through the second trimester. Some, such as *CYP11A1* and *17BHSD3*, which encode enzymes critical for androgen production, showed increased expression per testis over the period, whereas others, such as *CYP17A1*, showed no change. Similarly, there were no changes in expression of mRNA encoding key Leydig cell proteins such as INSL3 and LH receptor. Normalizing the data to Leydig cell number showed that there were no significant changes in any of the transcripts over the second trimester. Associated with these static transcript levels, there is a decline in both plasma and intratesticular testosterone over the latter half of the second trimester (this study and Ref. 9). Thus, toward the end of the second trimester, Leydig cell numbers and transcript levels are static, while testosterone is declining. Human fetal Leydig cell function in the second trimester is probably dependent on stimulation through the LH/chorionic gonadotropin receptor (44), and within this period serum levels of human chorionic gonadotropin (hCG) are reported to peak around 10–14 wk and decrease thereafter (45). It is not clear, however, that declining hCG levels are responsible for changes in testosterone because reported hCG levels remain very high throughout the second trimester (45) and would be expected to maintain steroidogenesis. Factors underlying the decline in androgen production during the second trimester remain to be determined, therefore, but may be related to maturational changes within the fetal Leydig cell population.

During embryogenesis the gonad and adrenal develop adjacent to each other on the medial side of the mesonephros and cell migration/expression patterns suggest that fetal adrenal and Leydig cells arise from the same pool of steroidogenic factor-1-positive progenitor cells (46). Further evidence linking these cells comes from recent mouse studies that have shown that: 1) fetal androgen production is sensitive to ACTH (47); 2) MC2R (the specific ACTH receptor) is expressed in fetal Leydig cells (48); 3) mRNA encoding adrenal enzymes CYP11B1 and CYP21A1 is also expressed in fetal Leydig cells (49, 59); and 4) the fetal adrenal expresses, or can be induced to express, mRNA encoding the testicular products CYP17A1 and LH receptor (50, 51). Our detection of *MC2R*, *CYP11B1*, and *CYP21A1* expression in the human fetal testis is further evidence of a link between fetal adrenal cells and fetal

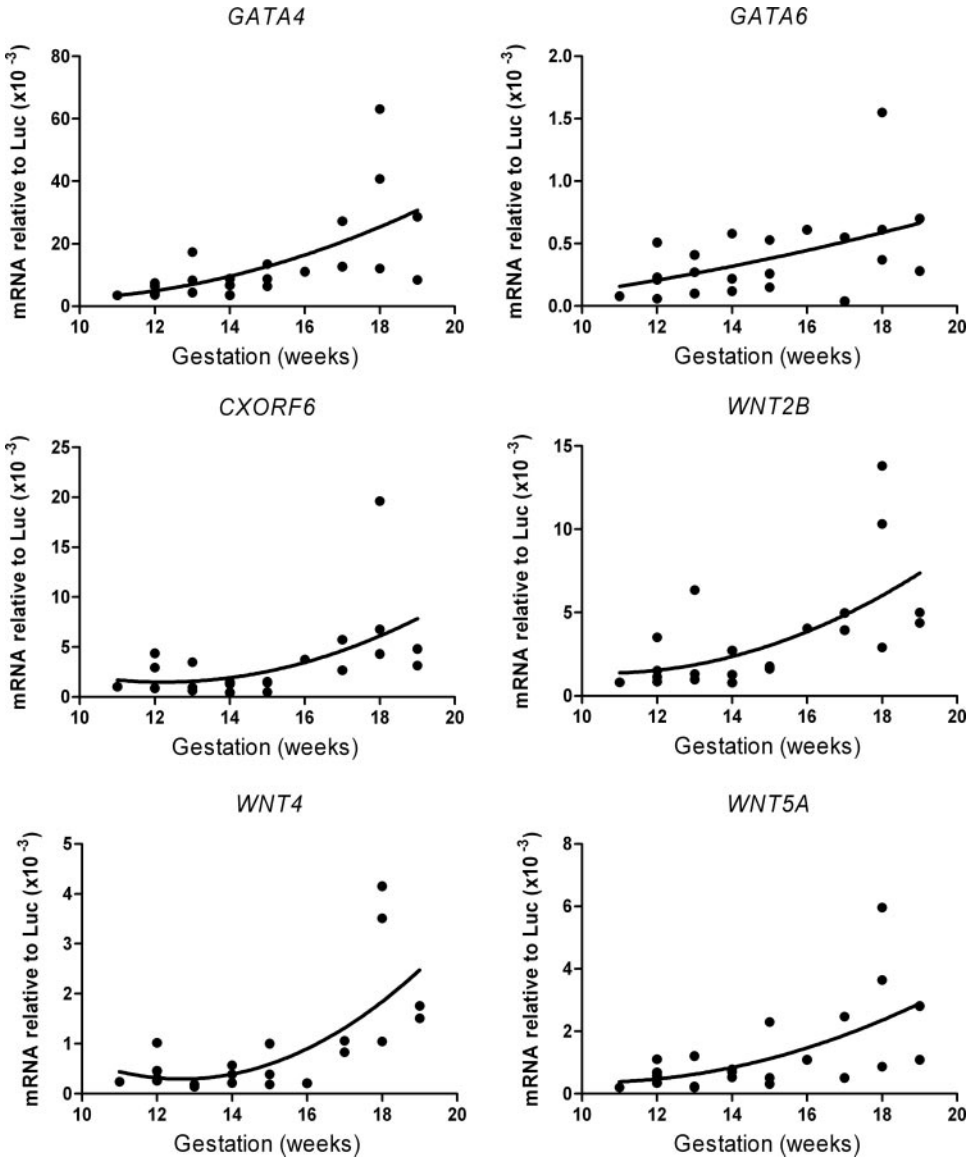

FIG. 6. Changes in levels of mRNA encoding transcription factors and developmental signaling genes in the human fetal testis during the second trimester (n = 22). RNA was extracted from testes at different ages and cDNA prepared as described in *Subjects and Methods*. Real-time PCR was used to measure cDNA levels relative to an external standard (luciferase) added during RNA extraction. Curves were fitted by nonlinear regression using a second-order polynomial. Individual data points are shown. Levels of all mRNA species measured increased significantly ($P < 0.05$) during the second trimester.

Leydig cells. However, the variable pattern of the expression makes it unlikely that ACTH plays a role in fetal Leydig cell function, unlike the mouse (47). Although it has been reported previously that MC2R is not expressed in the human fetal testis (52), our data are clearly indicative of MC2R expression. We are confident of these data because the PCR primers used crossed an intron/exon boundary, and sequencing of the amplicon confirmed the identity of the measured product. This apparent discrepancy may be related to the age of the sample used in the earlier study (8 wk) or, more simply, to the observed variability between individual fetuses.

The WNT proteins are signaling molecules involved in development. WNT4 is required for early Sertoli cell differentiation (53), but, in the mouse, expression is then inhibited in the developing testis to allow a normal male pattern vasculature to develop (54). In addition, WNT4 and FGF9 may act antagonistically in gonadal differentiation (40). WNT2B is involved in a number of developmental processes, including early kidney organogenesis (55). In contrast, WNT5A expression in the mouse testis is androgen regulated (43), suggesting that it may act as a mediator of androgen action. In our study, levels of mRNA encoding all three WNTs increased significantly through the second trimester. It is not clear whether this represents an increase or static per cell levels, but expression of WNT4 in the testis during the second trimester suggests that, beyond initial gonadal differentiation, it may play a role in later fetal testicular function as suggested in noneutherian mammals (56). Expression of the transcription factors *GATA4*, *GATA6*, and *CXORF6* also increased through the second trimester. There is good evidence that *GATA4* expression is required for Leydig cell differentiation (57), and continued expression through the second trimester may reflect an ongoing role in Leydig cell activity. The function of CXORF6 is unknown, but mutations in the gene lead to hypospadias, suggesting a critical role in fetal androgen production in the first or early second trimester

(30). Increasing expression through the second trimester would be consistent with a continuing role in Leydig cell function.

Overall, the present study characterizes second-trimester human testicular development. The main ongoing testicular event in the second trimester is continued rapid development of somatic and germ cell numbers that, in the case of the Sertoli cells and germ cells at least, is crucial for the establishment of subsequent adult fertility.

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