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The testicular transcriptome associated with spermatogonia differentiation initiated by gonadotrophin stimulation in the juvenile rhesus monkey (*Macaca mulatta*)

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STUDY QUESTION: What is the genetic landscape within the testis of the juvenile rhesus monkey (*Macaca mulatta*) that underlies the decision of undifferentiated spermatogonia to commit to a pathway of differentiation when puberty is induced prematurely by exogenous LH and FSH stimulation?

SUMMARY ANSWER: Forty-eight hours of gonadotrophin stimulation of the juvenile monkey testis resulted in the appearance of differentiating B spermatogonia and the emergence of 1362 up-regulated and 225 down-regulated testicular mRNAs encoding a complex network of proteins ranging from enzymes regulating Leydig cell steroidogenesis to membrane receptors, and from juxtacrine and paracrine factors to transcriptional factors governing spermatogonial stem cell fate.

WHAT IS KNOWN ALREADY: Our understanding of the cell and molecular biology underlying the fate of undifferentiated spermatogonia is based largely on studies of rodents, particularly of mice, but in the case of primates very little is known. The present study represents the first attempt to comprehensively address this question in a highly evolved primate.

STUDY DESIGN, SIZE, DURATION: Global gene expression in the testis from juvenile rhesus monkeys that had been stimulated with recombinant monkey LH and FSH for 48 h ($N = 3$) or 96 h ($N = 4$) was compared to that from vehicle treated animals ($N = 3$). Testicular cell types and testosterone secretion were also monitored.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Precocious testicular puberty was initiated in juvenile rhesus monkeys, 14–24 months of age, using a physiologic mode of intermittent stimulation with i.v. recombinant monkey LH and FSH that within 48 h produced 'adult' levels of circulating LH, FSH and testosterone. Mitotic activity was monitored by immunohistochemical assays of 5-bromo-2′-deoxyuridine and 5-ethynyl-2′-deoxyuridine incorporation. Animals were bilaterally castrated and RNA was extracted from the right testis. Global gene expression was determined using RNA-Seq. Differentially expressed genes (DEGs) were identified and evaluated by pathway analysis.

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mRNAs of particular interest were also quantitated using quantitative RT-PCR. Fractions of the left testis were used for histochemistry or immunoflouresence.

MAIN RESULTS AND THE ROLE OF CHANCE: Differentiating type B spermatogonia were observed after both 48 and 96 h of gonadotrophin stimulation. Pathway analysis identified five super categories of over-represented DEGs. Repression of *GFRA1* (glial cell line-derived neurotrophic factor family receptor alpha 1) and *NANOS2* (nanos C2HC-type zinc finger 2) that favor spermatogonial stem cell renewal was noted after 48 and 96 h of LH and FSH stimulation. Additionally, changes in expression of numerous genes involved in regulating the Notch pathway, cell adhesion, structural plasticity and modulating the immune system were observed. Induction of genes associated with the differentiation of spermatogonia stem cells (*SOHLH1* (spermatogenesis- and oogenesis-specific basic helix-loop-helix 1), *SOHLH2* and *KIT* (V-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog)) was not observed. Expression of the gene encoding STRA8 (stimulated by retinoic acid 8), a protein generally considered to mark activation of retinoic acid signaling, was below our limit of detection.

LARGE SCALE DATA: The entire mRNA data set for vehicle and gonadotrophin treated animals ($N = 10$) has been deposited in the GEO-NCBI repository (GSE97786).

LIMITATIONS REASONS FOR CAUTION: The limited number of monkeys per group and the dilution of low abundance germ cell transcripts by mRNAs contributed from somatic cells likely resulted in an underestimation of the number of differentially expressed germ cell genes.

WIDER IMPLICATIONS OF THE FINDINGS: The findings that expression of *GDNF* (a major promoter of spermatogonial stem cell renewal) was not detected in the control juvenile testes, expression of *SOHLH1*, *SOHLH2* and *KIT*, promoters of spermatogonial differentiation in mice, were not up-regulated in association with the gonadotrophin-induced generation of differentiating spermatogonia, and that robust activation of the retinoic acid signaling pathway was not observed, could not have been predicted. These unexpected results underline the importance of non-human primate models in translating data derived from animal research to the human situation.

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Key words: Rhesus monkey / spermatogonia / differentiation / global gene expression / testis / glial cell line-derived neurotrophic factor family receptor alpha 1 / RNA-Seq / gonadotrophin / spermatogenesis- and oogenesis-specific basic helix-loop-helix 1 / retinoic acid

Introduction

In man and monkey, spermatogenesis is initiated several years after birth by the increase in LH and FSH secretion that occurs with the onset of puberty (Plant *et al.*, 2005). During the juvenile phase of development, which is characterized by low circulating levels of gonadotrophins, the seminiferous cords of the monkey testis contain Sertoli cells (SCs) together with undifferentiated spermatogononia recognized as A-dark (Ad) and A-pale (Ap) spermatogonia (Plant *et al.*, 2005). These germ cells are present in approximately equal numbers and, as revealed by 5-bromo-2′-deoxyuridine (BrdU) labeling, both cell types proliferate in a gonadotrophin independent manner prior to puberty (Simorangkir *et al.*, 2005). Differentiating B spermatogonia, however, are only infrequently observed in the testis of the juvenile monkey (Plant *et al.*, 2005). Spermatogenesis in the juvenile monkey may be readily initiated with exogenous LH (or testosterone) and FSH (Arslan *et al.*, 1993; Ramaswamy *et al.*, 2000). Treatment of juvenile monkeys with an intermittent i.v. infusion of LH and FSH for 11 days resulted in the generation of germ cells as mature as leptotene spermatocytes (Ramaswamy *et al.*, 2000). If the kinetics of germ cell division leading to entry into meiosis in the adult are recapitulated when spermatogenesis is initiated prematurely in the juvenile with exogenous gonadotrophin, then it is reasonable to propose that the decision of undifferentiated spermatogonia to commit to a pathway of differentiation must be triggered very soon after the imposition of exogenous gonadotrophin stimulation. This is because in the adult testis the generation of leptotene spermatocytes from Ap requires approximately one cycle of the seminiferous epithelium, i.e. 10.5 days (Clermont, 1972; Simorangkir *et al.*, 2009)—the approximate duration of intermittent LH and FSH stimulation in the earlier study (Ramaswamy *et al.*, 2000).

Based on the foregoing considerations, we posited that if the duration of LH and FSH stimulation was limited to 48 h, the most mature germ cells in the juvenile testis at this time would be Ap that had made the commitment to differentiate. This being the case, it was further postulated that by comparing the transcriptome of the juvenile monkey testis after 48 h of stimulation with gonadotrophin versus that after vehicle administration it would be possible to identify the changes in gene expression associated with the decision of undifferentiated spermatogonia to commit to the pathway of differentiation. To this end, we employed RNA-Seq to establish global changes in gene expression in the juvenile monkey testis induced by 48 h of combined LH and FSH stimulation, and related differentially expressed genes (DEGs) to gonadotrophin-induced changes in the behavior of undifferentiated spermatogonia using S-phase labeling, standard morphometry and immunohistochemical evaluation of markers of differentiation. To determine the fate of those spermatogonia postulated to be committed to a pathway of differentiation after 48 h of gonadotrophin stimulation, the

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progeny of such cells and the associated change in the genetic landscape was assessed in a third group of monkeys in which gonadotrophin treatment was extended to 96 h.

Recombinant monkey LH and FSH administered i.v. in a pulsatile mode was used to mimic a physiological stimulation of the juvenile testis.

Materials and Methods

Animals

Ten juvenile male rhesus monkeys purchased from California National Primate Research Center, UC Davis, CA, USA were used. Six animals (14–24 months old, 2.4–3.6 kg body weight) were obtained in 2014 and four (14 months old, 2.0–2.8 kg body weight) in 2015. The animals were maintained under controlled photoperiod (lights on 07:00–19:00 h) and an ambient temperature of 21°C (Supplementary Information).

Experimental design

48 h LH and FSH or vehicle treatment: The six monkeys obtained in 2014 were used for this experiment. Four were 14–15 months old and two were 22–24 months old. The four younger animals formed two pairs while the two older animals formed a third pair. One animal in each of the three pairs was given a pulsatile iv gonadotrophin infusion while the other received vehicle (Supplementary Fig. S1). A bolus iv injection of 5-bromo-2′-deoxyuridine (BrdU) (33 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) was given 3 h prior to castration at 48 h.

96 h LH and FSH treatment: The four monkeys obtained in 2015 received the same gonadotrophin infusion as in the first experiment but the duration was increased to 96 h (Supplementary Fig. S1). These animals also received BrdU at 45 h but were not castrated until the end of the 96 h infusion. A bolus iv injection of 5-ethynyl-2′-deoxyuridine (EdU; 33 mg/kg body weight; Life Technologies, Grand Island, NY, USA) was given at 93 h, 3 h before castration.

Testicular volume and location were recorded at least once prior to infusion and, again, just prior to castration, as described previously (Ramaswamy *et al.*, 2000). For details of surgical procedures, gonadotrophin infusions, blood sampling, tissue processing and hormone assays see Supplementary Information.

RNA-seq, bioinformatics and RT-qPCR

RNA sequencing using an Illumina platform (Illumina, Inc, CA, USA) and the subsequent analysis of the reads generated was performed by the Genomics Research Core and Genomics Analysis Core, respectively; both at the University of Pittsburgh (Supplementary Information). The reference genome used was rheMac8 (BCM Mmul 8.0.1, obtained from NCBI RefSeq database).

To identify the DEGs after either 48 or 96 h of LH and FSH treatment, as well as those emerging between 48 and 96 h of gonadotrophin stimulation (termed ‘transitional’ DEGs), pairwise differential expression using exact test (McCarthy *et al.*, 2012) was performed with edgeR (v. 3.8.6) (McCarthy *et al.*, 2012; Anders *et al.*, 2013; Zhou *et al.*, 2014). DEGs were defined as those having normalized counts per million mapped reads ≥ 1 for at least two of three samples (48 h) or three of four (96 h), false discovery rate (FDR) values < 0.05 ($<5.0E-2$) and a mean fold change (FC) $\geq$ 50% (FC ≥ 1.5 or ≤ 0.5, rounded to the nearest 10th).

RT-qPCR was performed as described in Supplementary Information using the primers listed in Supplementary Table SI.

Histology, morphometry and S-phase labeling

For histomorphological and BrdU labeling analyses, five 5 μm sections of Bouin’s fixed testis from each animal and collected at intervals of at least 100 μm, were stained with either periodic acid-Schiff-hematoxylin (PAS-H kit, Sigma Chemical Co., St. Louis, MO, USA) or BrdU (Supplementary Information) and PAS-H, respectively. Estimation of number of SCs and undifferentiated (Ad and Ap) spermatogonia per testis was performed by a blinded evaluator (G.R.M.), as described previously (Marshall and Plant, 1996). B spermatogonia were also expressed as number per testis. BrdU labeling indices were calculated as described in Supplementary Information.

Immunofluorescence histochemistry

Expression of glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1), and spermatogenesis- and oogenesis-specific basic helix-loop-helix 1 (SOHLH1) was determined using fluorescence immunohistochemistry as described in detail previously (Ramaswamy *et al.*, 2014) (Supplemental Information).

For detection of dual S-phase labeled cells, BrdU was first visualized with the anti-BrdU antibody as described previously (Ramaswamy *et al.*, 2014). EdU labeling was then performed according to the protocol supplied with the manufacturer’s kit (Click-IT EdU imaging kit, C10337, Invitrogen, Carlsbad, CA, USA) that uses Alexa Fluor 488 for detection. For the negative control for BrdU, the primary antibody was omitted, as previously described (Ramaswamy *et al.*, 2014). For the negative control for EdU, sections from the testis of a non-EdU treated juvenile monkey were used (Supplementary Fig. S2). Details of secondary antibodies are provided in Supplementary Table SII.

Statistical analysis

For determination of the significance of differences between groups in cell number, labeling index, relative integrated density and mRNA levels determined by qRT-PCR, the Student’s *T*-test or one-way ANOVA were employed as appropriate (Prism 5, GraphPad Software, Inc., La Jolla, CA, USA). Significance was determined when $P \leq 0.05$. Data for these parameters are expressed as the mean $\pm$ SEM.

Ethical approval

The experiments were conducted in accordance with NIH Guidelines for the Care and Use of Experimental Animals and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Results

Endocrine response of the testis to LH and FSH stimulation

Administration of intermittent iv infusions of LH and FSH to juvenile monkeys for 48 or 96 h resulted in a corresponding pulsatile pattern in the concentrations of the circulating gonadotrophins and increases in testosterone secretion that reached adult levels by 48 h (Supplementary Fig. S3). Increases in many LH and FSH inducible genes, including a >10-fold up-regulation in the mRNA encoding steroidogenic acute regulatory protein (STAR), were noted (Supplementary Table SIII).

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Testicular growth in response to LH and FSH stimulation

Mean testicular volumes before initiation of the infusions were 0.66 ± 0.1 ml (vehicle), 0.75 ± 0.1 ml (48 h) and 0.51 ± 0.1 ml (96 h). Combined testicular weights in the three groups were 0.86 ± 0.16, 1.12 ± 0.03 and 0.96 ± 0.12 g (vehicle, 48 and 96 h gonadotrophin, respectively). Mean diameter of the seminiferous cord after 48 h of gonadotrophin (50.5 ± 1.7 μm) was significantly greater ($P < 0.05$) than vehicle (45.3 ± 2.2 μm). After 96 h of gonadotrophin stimulation, a diameter of 53.5 ± 4.1 μm was noted. Testicular descent was initiated in most monkeys by gonadotrophin stimulation (Supplementary Information).

Cellular response to LH and FSH stimulation

In monkeys that received vehicle, B spermatogonia were not observed but, importantly, these differentiating spermatogonia were observed in two of three animals after stimulation with gonadotrophin for 48 h and in three of four monkeys after 96 h of stimulation (Fig. 1). The mean number of B spermatogonia per testis after 48 and 96 h of gonadotrophin

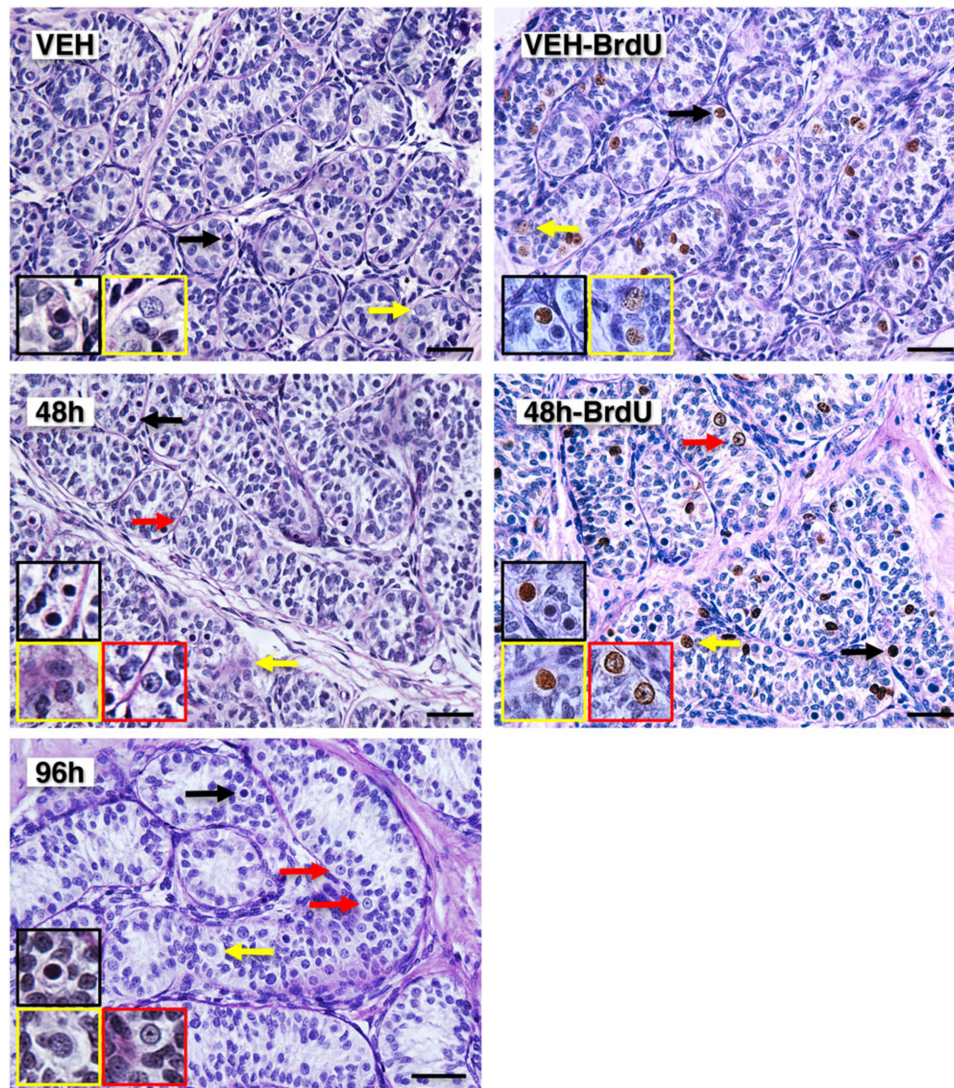

Figure 1 Morphology and mitotic labeling of seminiferous cords in the juvenile rhesus monkey following LH and FSH stimulation. Photomicrographs of 5 μm sections from the testis of juvenile male rhesus monkeys following vehicle treatment (VEH; top panels) or gonadotrophin stimulation for 48 h (middle panels) or 96 h (bottom panel). Sections on the left were stained with periodic acid-Schiff-hematoxylin (PAS-H). Sections on the right were used to immunohistochemically visualize 5-bromo-2′-deoxyuridine (BrdU, brown) and counter stained with PAS-H. Insets in each panel show at higher magnification the germ cells indicated by arrows in the respective photomicrograph: Black arrow, Ad spermatogonia; yellow arrow, Ap spermatogonia; red arrow, B spermatogonia. Note that the cells with BrdU labeled nuclei in the right hand panels. BrdU labeling at 96 h is not shown because the S-phase marker was injected 51 h prior to castration rather than 3 h before removal of the testis as was the case for the 48 h gonadotrophin and vehicle infusions. Scale bar, 50 μm.

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stimulation was $0.8 \pm 1.0 \times 10^6$ and $0.6 \pm 0.4 \times 10^6$, respectively. The majority of B spermatogonia observed after 48 h of gonadotrophin stimulation were BrdU labeled. In all four monkeys that received gonadotrophin for 96 h, immunofluorescence detection of the S-phase markers, BrdU and EdU (administered at 45 and 93 h, respectively, after the start of the infusion), revealed dual labeled cells within the semiferous cords in ~10% of the fields examined. These dual labeled cells were generally located on the basement membrane, frequently found in pairs, and exhibited large DAPI stained spherical nuclei with a homogeneous and relatively translucent appearance characteristic of spermatogonia (Fig. 2). The effect of gonadotrophin stimulation on the numbers and labeling indices (BrdU) of SCs and undifferentiated Ad and Ap spermatogonia are presented in Supplementary Fig. S4.

The testicular transcriptome of the juvenile monkey

RNA-Seq of testicular RNA from the vehicle and gonadotrophin treated juvenile monkeys ($N = 10$) identified 26813 transcript assemblies (GSE97786; GEO-NCBI repository). Filtering of the transcript assemblies for the three vehicle treated monkeys, as described in Supplemental Information, resulted in identification of 15475 genes of which 15174 were protein encoding or pseudogenes. The testicular

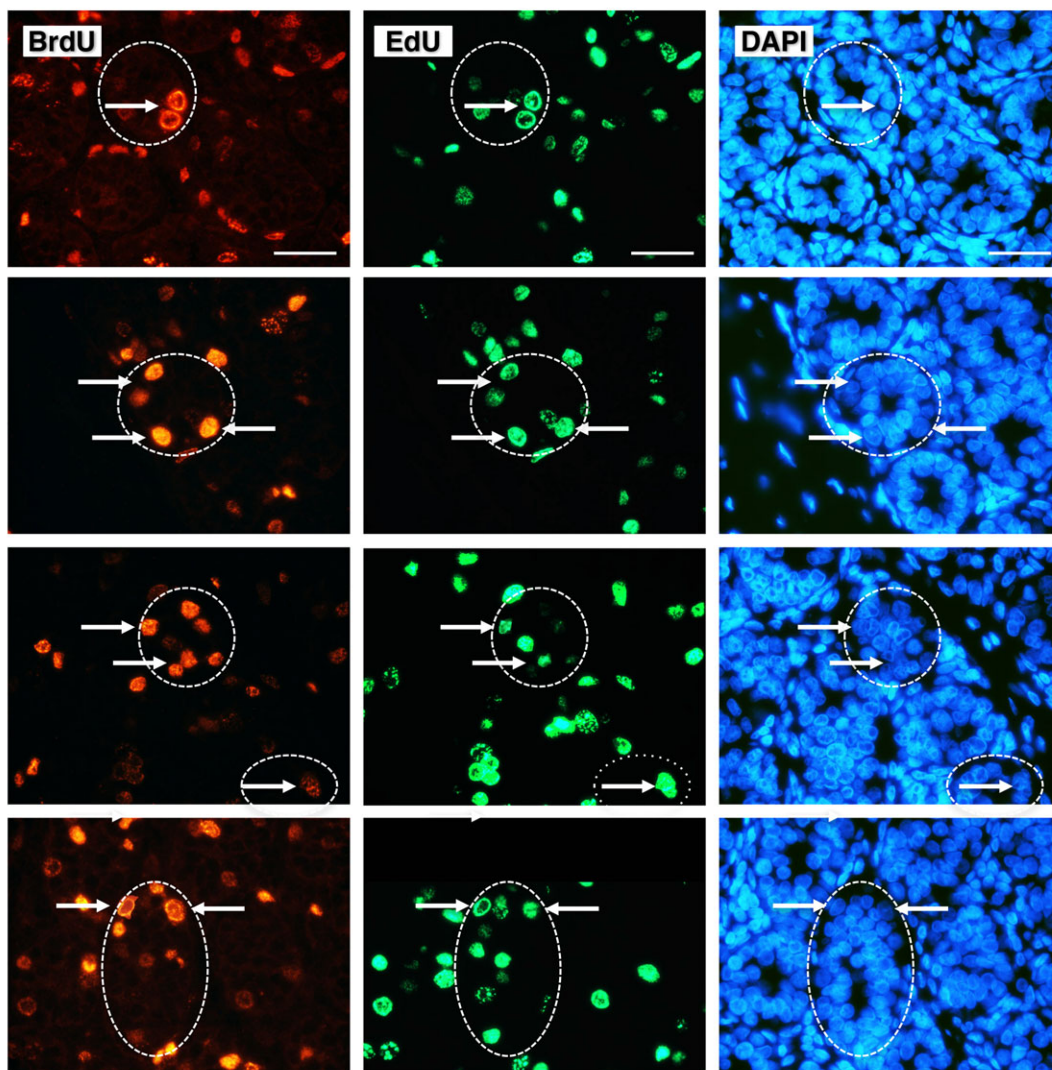

Figure 2 Spermatogonia stimulated with LH and FSH for 96 h are S-phase labeled at both 45 h (BrdU) and 93 h (Edu). Photomicrographs of immunofluorescence staining for BrdU (left panels), 5-ethynyl-2′-deoxyuridine (EdU, middle panels) and DAPI (right panels) in 5 μm sections from the testis of each of the four juvenile male rhesus monkeys that received 96 h of LH and FSH stimulation and were given an iv bolus of BrdU and EdU at 45 and 93 h, respectively, after the start of the gonadotrophin infusion. Castration was conducted at the termination of the infusion (96 h). Note that the dual BrdU + EdU labeled cells (arrows) located inside the seminiferous cords delineated by the dotted profiles. Some of the dual labeled cells were tentatively identified as clones of differentiating B spermatogonia (e.g. top panels). Scale bar, 50 μm.

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transcriptome of the juvenile monkey is provided in Supplementary Table SIV.

Differential gene expression induced by 48 h of LH and FSH stimulation

Overview

Forty-eight hours of gonadotrophin stimulation of the juvenile testis resulted in the emergence of 1587 DEGs (1362 up-regulated, 225 down-regulated) relative to 48 h of vehicle administration, and a further 48 h of exposure to LH and FSH increased the number of DEGs relative to vehicle to 2117 (1604 up-regulated, 514 down-regulated). Gonadotrophin stimulation for either 48 or 96 h resulted in 915 shared DEGs, leaving 58% of the DEGs at 96 h (1218) unique to the later time point. Expression of the majority of the 915 DEGs common to both periods of gonadotrophin stimulation did not change during the last 48 h of LH and FSH exposure. When gene expression after 48 h of stimulation was compared to that after 96 h of LH and FSH treatment, 379 DEGs were identified. Of these 'transitional' DEGs, 238 were up-regulated and 61 were down-regulated. The DEGs at 48 and 96 h of gonadotrophin stimulation and the transitional DEGs are provided in Supplementary Table SVa-c. On the 11 occasions when RT-qPCR was used to confirm RNA-Seq data for key genes (Supplementary Table SI), the two methods provided congruent results.

Ingenuity pathway analysis of the up-regulated DEGs after 48 and 96 h of gonadotrophin treatment identified 62 and 63 over-represented pathways, respectively, many of which could be grouped into five super categories related to cell proliferation and metabolism (Supplementary Table SVIa and b). For the transition DEGs, six pathways related to cell proliferation and signal transduction were identified (Supplementary Table SVIc).

Table I Genes implicated in spermatogonia differentiation and their changes in expression in the juvenile monkey testis after 48 or 96 h of gonadotrophin stimulation.

Gene*	FC 48 h GTH versus VEH	FDR 48 h GTH versus VEH	FC 96 h GTH versus VEH	FDR 96 h GTH versus VEH
BMP4	0.51	0.02	0.56	0.03
DMRT1	1.76	<0.01	1.19	0.42
KIT	0.66	0.13	0.53	<0.01
NGN3	Not detected		Not detected	
NKAPL	0.76	0.50	0.55	0.03
PHF13, SPOC1	0.88	0.64	0.90	0.61
SALL4	0.85	0.73	0.64	0.12
SMC6	1.08	0.79	1.28	0.18
SOHLH1	0.75	0.39	0.47	<0.01
SOHLH2	0.76	0.44	0.67	0.13
SOX3	Not detected		Not detected	
STAT3	1.27	0.24	1.30	0.11
TEX14	0.84	0.65	0.65	0.10

*See Supplementary Table SIV for full name of all genes.
GTH, gonadotrophic hormones; VEH, vehicle; FC, fold change; FDR, false discovery rate.
DEGs highlighted in RED are up-regulated and in GREEN are down-regulated.

Germ cell genes regulating spermatogonial fate

Surprisingly, few germ cell genes recognized to be associated with determining spermatogonial stem cell fate in the rodent were differentially expressed in the testis of the juvenile monkey in response to stimulation with LH and FSH (Tables I and II). Of the spermatogonia-specific genes posited to favor differentiation, neither *SOHLH1* nor *SOHLH2* that encode transcription factors nor their downstream target *KIT* (V-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) (Rossi, 2013), a gene encoding a cell surface tyrosine-protein kinase receptor, were induced (Table I). Furthermore, immunofluorescence studies detected no increase in nuclear localization of SOHLH1 in gonadotrophin stimulated testes (Supplementary Fig. S5).

In the case of germ cell genes generally viewed as favoring spermatogonial stem cell renewal (Table II), *GFRA1* and *ZBTB16* (zinc finger and BTB domain containing 16; also called *PLZF* [promyelocytic leukemia zinc finger]) were both down-regulated by > 50% after 48 h of gonadotrophin stimulation (Fig. 3). The repression of *GFRA1* was sustained at 96 h of stimulation and this was associated with decreased expression of *NANOS2* (nanos C2HC-type zinc finger 2), a gene that encodes an RNA-binding protein that promotes the renewal of spermatogonial stem cells (Zhou *et al.*, 2015). Overall GFRA1 immunoreactivity was decreased, although the number of GFRA1 positive cells did not change with gonadotrophin treatment (Fig. 4). Known downstream target genes of GFRA1 (Schmidt *et al.*, 2009) and RET (ret proto-oncogene), the gene that encodes the co-receptor of GFRA1 (Hofmann, 2008), were not differentially expressed. The values for the mRNA encoding glial cell-derived neurotrophic factor (GDNF), a ligand for GFRA1 (Hofmann, 2008), did not reach the criteria for an expressed gene, even in the vehicle treated animals. In rodents, GDNF signaling via GFRA1 is required for maintaining spermatogonial stem cell self-renewal (Meng *et al.*, 2000), and we had anticipated that *GDNF* expression would be high in the vehicle treated juvenile where the population of undifferentiated spermatogonia is expanding in an insidious manner (Simorangkir *et al.*, 2005).

Genes encoding paracrine and juxtacrine signals regulating spermatogonial fate

Retinoic acid (RA) signaling: *RDH10* (retinol dehydrogenase 10) was up-regulated approximately 2-fold after 48 or 96 h of gonadotrophin stimulation, and expression of *CRABP2* (cellular retinoic acid-binding protein 2) was up-regulated 4-fold after 96 h (Supplementary Table SVII). However, the gene encoding alcohol dehydrogenase 7 (*ADH7)* was down-regulated by more than 50% after both 48 and 96 h of gonadotrophin stimulation and expression of STRA8 (stimulated by retinoic acid 8), a well-known marker for RA pathway activation was too low to be assessed (Supplementary Table SVII).

NOTCH signaling: Gonadotrophin stimulation of the juvenile testis for 48 h and 96 h resulted in an ~3-fold up-regulated expression of *DLK1* (delta like non-canonical Notch ligand 1) and this was associated,

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Table II Genes implicated in spermatogonia stem cell renewal and their changes in expression in the juvenile monkey testis after 48 or 96 h of gonadotrophin stimulation.

Gene*	FC 48 h GTH versus VEH	FDR 48 h GTH versus VEH	FC 96 h GTH versus VEH	FDR 96 h GTH versus VEH
CCR1	1.78	0.36	1.61	0.39
CD24	0.50	0.12	0.54	0.11
CD9	1.29	0.45	1.03	0.94
CDH1	1.39	0.44	0.51	0.02
CSFR1	Not detected		Not detected	
CXCR4	1.30	0.56	0.87	0.74
DAX1	Not detected		Not detected	
NROB1	Not detected		Not detected	
DAXX	1.06	0.85	0.98	0.93
EGR3	1.43	0.64	1.17	0.84
EPCAM (TACSTD1)	1.11	0.86	0.75	0.39
ERBB3	0.50	<0.01	0.30	<0.01
EZH2	1.29	0.22	1.89	<0.01
FGFR1	0.76	0.35	1.11	0.72
FGFR2	1.07	0.88	1.73	0.02
FGFR3	0.75	0.24	0.51	<0.01
FGFR4	0.84	0.69	0.51	0.01
FOXO1	0.64	<0.01	0.81	0.16
GFRA1	0.31	<0.01	0.49	<0.01
GPR125	Not detected		Not detected	
ID4	0.69	0.09	0.68	0.04
IGFBP3	0.11	<0.01	0.21	<0.01
ITGA6	0.99	0.99	0.81	0.26
ITGB1	1.20	0.35	1.31	0.07
LHX1	Not detected		Not detected	
Lin28B	0.75	0.33	0.68	0.08
MAGEA4	0.84	0.66	0.57	0.02
NANOS2	0.52	0.25	0.26	<0.01
NANOS3	0.67	0.35	0.65	0.21
PAX7	0.41	0.19	0.56	0.32
PIWIL2	1.05	0.86	1.17	0.27
POU3F1	1.06	0.96	0.65	0.34
PTEN	1.27	0.30	1.49	0.02
PTPN11 (SHP2)	1.45	0.02	1.38	0.03
RB1	1.63	<0.01	1.91	<0.01
RCOR2	1.01	0.99	0.83	0.25
RET	0.98	0.97	0.99	0.98
T (brachyury)	Not detected		Not detected	
TAF4B	0.96	0.91	0.89	0.56
THY1	2.80	<0.01	4.77	<0.01
TP53	1.76	<0.01	1.57	<0.01
UCHL1 (PGP9.5)	0.90	0.75	0.59	0.01
UTF1	Not detected		Not detected	
ZBTB16 (PLZF)	0.36	<0.01	0.60	0.15

*See Supplementary Table SIV for full name of all genes.
DEGs highlighted in RED are up-regulated and in GREEN are down-regulated.

particularly at 96 h, with changes in expression of several genes considered to encode either targets or effectors of NOTCH signaling (Supplementary Table SVIII). Expression of genes encoding the NOTCH receptors was not influenced by gonadotrophin treatment. The gene encoding NF-κB activating protein like (*NKAPL*), a posited suppressor of NOTCH signaling, was down-regulated at 96 h.

Genes implicated in regulating stem cell fate in other systems

Several genes previously recognized to be expressed by, and/or determine the fate of stem cells in tissues other than testis were identified as DEGs in the present study (Table III), suggesting perhaps previously unrecognized roles of such genes in governing the behavior of undifferentiated spermatogonia.

Adhesion, cytoskeletal and extra-cellular matrix genes

A notable number of genes encoding proteins considered to be important in regulating cell movement, structural plasticity, establishment of the blood-testis barrier and spermatogonial stem cell niche were differentially expressed (predominantly up-regulated) following either 48 or 96 h, and in most cases at both periods of gonadotrophin stimulation (Table IV). These included claudin 11 (CLDN11), several actins and actin related proteins, plus a variety of collagens, annexins, tubulins and laminins.

Immune genes

Several genes that play a role in immunity or inflammation displayed a significant change in expression following gonadotropin secretion. Notably, several genes that encode proteins that are produced by macrophages or regulate the recruitment of these immune cells to the testis were up-regulated (Supplementary Table SIX).

Discussion

As outlined in the Introduction, we posited that LH and FSH stimulation of the juvenile testis for 48 h would be sufficient to drive undifferentiated Ap spermatogonia to commit to a pathway of differentiation, but would be of insufficient duration for such Ap spermatogonia to

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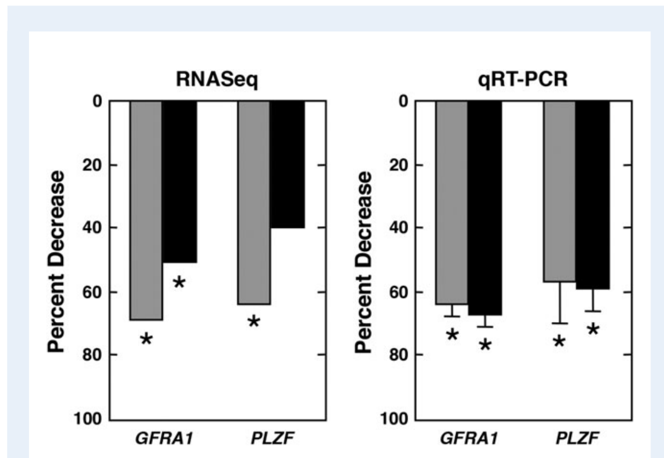

Figure 3 LH and FSH stimulation of the juvenile monkey testis suppresses the expression of *GFRA1* and *PLZF*. Percentage decrease (mean ± SEM) in stemness associated *GFRA1 (*glial cell line-derived neurotrophic factor family receptor alpha 1) and *ZBTB16* (zinc finger and BTB domain containing 16; also called *PLZF* [promyelocytic leukemia zinc finger]) gene transcripts as determined by RNA-Seq (left panel) and RT-qPCR (right panel) in testis from juvenile rhesus monkeys following 48 h (gray bars) or 96 h (black bars) of gonadotrophin stimulation. Data normalized to vehicle-treated group. *, $P \leq 0.05$ versus vehicle group, *T*-test). $N = 3$ or 4.

complete a differentiating mitosis and produce the first generation of B spermatogonia, a step in the spermatogenic lineage that we anticipated would require extending the duration of gonadotrophin stimulation to 96 h. This prediction was partially realized. B spermatogonia were not observed in the vehicle treated testis but, in contrast to our expectation, these differentiating spermatogonia were observed in two of the three animals that received 48 h of gonadotrophin stimulation. B spermatogonia were also observed in three of the four monkeys that received 96 h of gonadotrophin stimulation but the duration of treatment did not influence the number of differentiating B spermatogonia. This result may be explained by the noticeably smaller size of the testes at the start of the experiment in the monkeys that received the 96 h infusion. Regardless, it may be concluded that after 48 h of gonadotrophin stimulation the testes of the juvenile monkeys contained a cohort of Ap spermatogonia that, as a result of the action of LH and FSH, had committed to the pathway of differentiation. This view is further supported by the finding that after 96 h of gonadotrophin treatment we observed germ cells dual labeled for BrdU and EdU, S-phase markers that had been administered 48 h apart during LH and FSH stimulation. If the duration of the cell cycle of Ap and B spermatogonia in the juvenile monkey testis are the same as those in the adult testis, i.e. ~11 and 2 days, respectively, (Clermont, 1972; Simorangkir *et al.*, 2009), a cell that completed an entire cell cycle in 48 h (i.e. a cell with both S-phase labels) could not be a proliferating/ renewing Ap spermatogonia. Rather, the dual labeled cells at 96 h

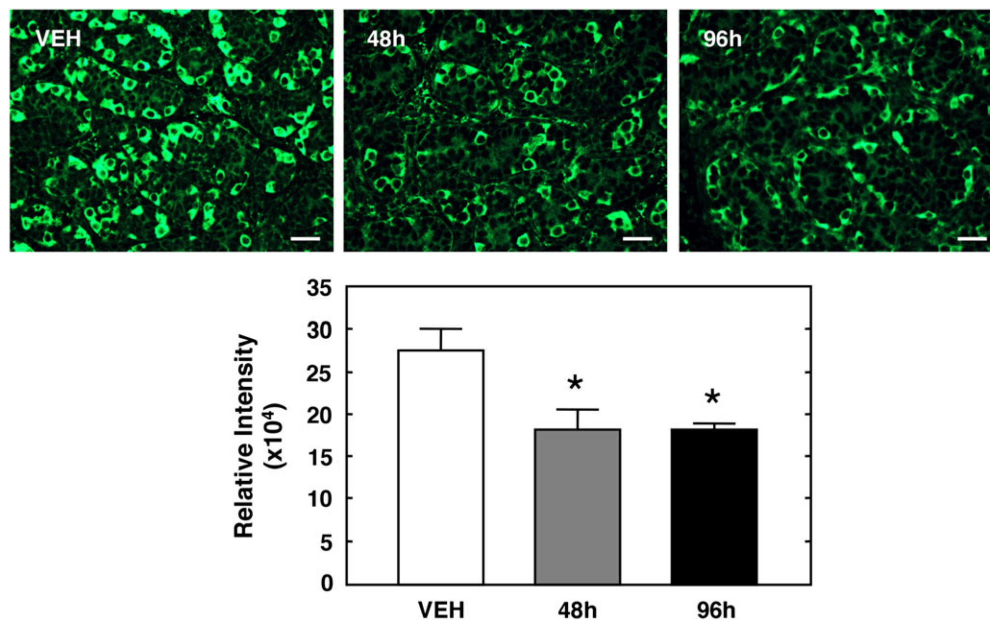

Figure 4 Suppression of GFRA1 immunofluorescence in the testis of the juvenile monkey following LH and FSH stimulation. Photomicrographs of 5 μm sections from the testis of a juvenile rhesus monkey showing immunofluorescence staining for GFRA1 positive spermatogonia following vehicle treatment (VEH; top left panel) or gonadotrophin stimulation for 48 h (top middle panel) or 96 h (top right panel). The lower panel shows quantification using imageJ software of the relative integrated density (mean ± SEM) of the GFRA1 signal in spermatogonia in the three groups (VEH, open bar; 48 h, gray bar; 96 h, black bar). Scale bar, 50 μm; *$P \leq 0.05$ compared to VEH, one-way ANOVA). $N = 3$ or 4.

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Table III Genes implicated in regulation of stem cell fate in non-testicular tissue and their changes in expression in the juvenile monkey testis after 48 or 96 h of gonadotrophin stimulation.

Gene*	FC 48 h GTH versus VEH	FDR 48 h GTH versus VEH	FC 96 h GTH versus VEH	FDR 96 h GTH versus VEH	Function	References
FDZ7	0.47	<0.01	0.55	<0.01	WNT signaling protein	Melchior *et al.* (2008); Fernandez *et al.* (2014); Mei *et al.* (2014)
GDF5	0.55	0.23	0.16	<0.01	TGF-beta family ligand	Tan *et al.* (2015); Colombier *et al.* (2016)
KCNS3	0.24	<0.01	0.53	0.17	Ion channel regulator	Sandberg *et al.* (2014)
NACC1	1.92	<0.01	1.64	0.02	Transcriptional repressor	Kim *et al.* (2008)
NME2	1.70	<0.01	1.49	<0.01	Transcriptional activator of the MYC gene	Zhu *et al.* (2009); Nieto-Estevez *et al.* (2013)
NDRG1	1.86	<0.01	1.92	<0.01	N-myc down-regulated gene family member	Zhu *et al.* (2009); Nieto-Estevez *et al.* (2013)

*See Supplementary Table SIV for full name of all genes.
DEGs highlighted in RED are up-regulated and in GREEN are down-regulated.

must have been differentiating B spermatogonia, the parents of which were most likely Ap spermatogonia.

It should be noted that the above ideas are based on the classical model of Clermont for the kinetics of spermatogenesis in the adult monkey, where Ad spermatogonia are viewed as reserve spermatogonial stem cells that rarely divide (Clermont, 1972). In the juvenile monkey, however, Ad spermatogonia also are proliferating, as shown in this and an earlier study (Simorangkir *et al.*, 2005). Moreover, the progeny of Ad spermatogonia division in the juvenile, and any impact of gonadotrophin stimulation on these cells, has not been established. Therefore, it is possible that Ad, in addition to Ap, contribute to the cohort of spermatogonia that commit to differentiation.

The analysis of global gene expression in the intact testis under physiological conditions is a major strength of the present study. However, one consequence of this approach is that the number of genes identified to be associated with the decision of undifferentiated spermatogonia to commit to a pathway of differentiation is likely to be underestimated. This is because prior to gonadotrophin stimulation of the juvenile monkey testis these germ cells comprise approximately only 10% of the seminiferous chords. Thus, mRNAs contributed from somatic cells are likely to compromise detection of low abundance germ cell transcripts. One other aspect of experimental design that should be noted is that the monkeys studied ranged from 14 to 24 months of age. The entire juvenile phase of development in this species unfolds in a hypogonadotrophic and hypoandrogenic state and neither the hormonal status nor the qualitative cellular composition of the testis changes noticeably during this period (Plant *et al.*, 2005). Therefore, it is reasonable to assume that the genetic landscape of the juvenile testis is similar at 14 and 24 months of age.

Germ cell genes that underpin spermatogonial fate in mammals have been investigated almost exclusively in rodents; particularly in mouse models (Valli *et al.*, 2015). In the present study, expression of *GFRA1*, a gene encoding a cell surface receptor that in co-operation with the associated RET tyrosine kinase, activates intracellular pathways in undifferentiated spermatogonia that promote self-renewal and proliferation (Hofmann, 2008), was down-regulated by 48 and 96 h of LH and FSH stimulation. Moreover, the decrease in testicular *GFRA1* mRNA levels was associated with a decline in expression of the encoded protein in undifferentiated A spermatogonia. Although studies indicate that *GFRA1* mRNA levels can be down-regulated by SOHLH1 or SOHLH2 in mouse testes (Suzuki *et al.*, 2012), expression of the mRNAs encoding these transcription factors were not increased after gonadotrophin treatment in the present study. Moreover, translocation of SOHLH1 to the spermatogonial nucleus, which in the monkey occurs during spontaneous puberty in association with the appearance of differentiating B spermatogonia (Ramaswamy *et al.*, 2014), was not observed. Thus, the SOHLHs do not appear to be responsible for the gonadotrophin-induced decrease in expression of *GFRA1* observed in the present study.

Although few genes identified as downstream targets of GFRA1 (Schmidt *et al.*, 2009) emerged as DEGs following gonadotrophin stimulation, expression of *NANOS2*, which is down-regulated in *Gfra1* null mice (Sada *et al.*, 2012), was reduced after both 48 h and 96 h of LH and FSH stimulation. Since this zinc finger RNA-binding protein has been shown to suppress differentiation of spermatogonial stem cells in the mouse (Sada *et al.*, 2012), it would seem reasonable to propose that, in the monkey, diminished GFRA1-NANOS2 signaling in undifferentiated A spermatogonia may represent a key pathway underlying the initial decision of these cells to commit to the pathway of differentiation.

The gonadotrophin-induced down-regulation of *GFRA1* expression was associated with a reduction in the level of *ZBTB16* mRNA, which encodes a zinc finger transcription factor that, like GFRA1, has also been implicated in the maintenance of the spermatogonial stem cell population in rodents (Hobbs *et al.*, 2010). Although there is evidence for ZBTB16 induced repression of the *Kit* gene (Filipponi *et al.*, 2007), the view that KIT signaling in these cells is primarily governed by post-transcriptional mechanisms has recently been proposed (Busada *et al.*, 2015). Regardless, increases in *KIT* mRNA levels were not observed in the juvenile monkey testis following gonadotrophin stimulation and therefore an up-regulation in expression of this gene did not appear to account for the initial decision of undifferentiated A spermatogonia to commit to a pathway of differentiation in the present model. The data for *KIT* are consistent with the absence of gonadotrophin-induced changes in the expression of the genes encoding SOHLH1 and SOHLH2 (see above) that are considered upstream regulators of *KIT* expression (Rossi, 2013).

The gonadotrophin-dependent signals underlying the down-regulation in expression of *GFRA1*, *NANOS2* and *ZBTB16*, were presumably relayed to the germ cells indirectly by somatic cells of the

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Table IV Changes in expression of adhesion, cytoskeletal and extra-cellular matrix genes in the juvenile monkey testis after 48 or 96 h of gonadotrophin stimulation.

Gene*	FC 48 h GTH versus VEH	FDR 48 h GTH versus VEH	FC 96 h GTH versus VEH	FDR 96 h GTH versus VEH
ACTA2	6.80	<0.01	5.18	<0.01
ACTG1	1.45	0.01	1.45	<0.01
ACTR1A	2.35	<0.01	1.87	<0.01
ACTR2	1.64	<0.01	1.65	<0.01
ACTR3	1.60	<0.01	1.42	0.01
ADAM33	0.51	0.03	0.83	0.59
ADAMTS2	1.80	0.06	2.41	<0.01
ADAMTS9	3.37	<0.01	3.25	<0.01
ANGPT1	0.42	<0.01	0.46	<0.01
ANXA1	1.75	0.12	2.31	<0.01
ANXA2	1.81	0.01	1.95	<0.01
ANXA11	1.63	0.01	1.55	0.02
ARPC1B	2.45	<0.01	1.74	<0.01
ARPC5L	1.57	0.01	1.29	0.14
BCAM	0.43	<0.01	0.54	<0.01
CAPG	1.89	0.01	2.15	<0.01
CDH1	1.39	0.44	0.51	0.02
CFL1	2.00	<0.01	1.48	<0.01
CLDN11	2.51	<0.01	2.41	<0.01
CNTNAP4	0.24	0.01	0.39	0.04
COL16A1	1.68	0.08	2.17	<0.01
COL17A1	1.13	0.79	0.86	0.64
COL1A1	2.55	0.06	5.71	<0.01
COL4A3	0.39	0.03	0.46	0.04
COL6A3	1.81	0.07	3.13	<0.01
COL9A1	0.41	<0.01	0.56	<0.01
ECM1	1.67	<0.01	1.98	<0.01
EDN1	0.39	0.01	0.28	<0.01
ERBB3	0.45	<0.01	0.29	<0.01
FN1	2.27	0.01	3.30	<0.01
ITGA10	0.35	<0.01	0.68	0.32
LAMA1	1.68	0.03	1.44	0.12
MYL6	1.80	<0.01	1.31	0.05
MYL6B	2.32	<0.01	1.55	<0.01
NID2	3.11	<0.01	3.99	<0.01
NRCAM	0.35	<0.01	0.36	<0.01
NTN4	0.51	<0.01	0.35	<0.01
PVRL3	1.77	<0.01	2.20	<0.01
RASL11A	0.41	<0.01	0.47	<0.01
SDC3	2.99	<0.01	2.85	<0.01
SEPT6	1.73	0.15	1.15	0.75
SPP1	5.69	<0.01	0.63	0.52

Continued

Table IV *Continued*

Gene*	FC 48 h GTH versus VEH	FDR 48 h GTH versus VEH	FC 96 h GTH versus VEH	FDR 96 h GTH versus VEH
TUBA1C	Not detected		Not detected	
TUBB	2.80	<0.01	2.42	<0.01
TUBB6	Not detected		Not detected	

*See Supplementary Table SIV for full name of all genes.
DEGs highlighted in RED are up-regulated and in GREEN are down-regulated.

testis. The SC expresses both FSH and androgen receptors (Smith and Walker, 2015) and is therefore capable of integrating both the LH signal (indirectly via testosterone secretion from the Leydig cell) and FSH signal. Thus, this somatic cell would be expected to provide a major hub that provides the final link to the germ cell in the form of a paracrine or juxtacrine signal.

All-*trans* RA that is generated from circulating vitamin A (retinol) after transport across the plasma membrane of the SC is recognized as an important paracrine signal required for the transition in rodents between Aal and early differentiating spermatogonia (A1–A4 spermatogonia) (Griswold, 2016). Two components of the RA signaling pathway, *RDH10* and *CRABP2*, were up-regulated after gonadotrophin stimulation. However, the failure to detect *STRA8* mRNA, a recognized marker of activation of RA signaling, raises the possibility that this pathway is not brought into play in the juvenile monkey testis until after 96 h of gonadotrophin stimulation.

It is reasonable to propose that paracrine signals dictating spermatogonial fate are likely to be co-ordinated with juxtacrine cues to generate complimentary molecular and structural environments underlying decisions by germ cells to differentiate or proliferate. In this regard, NOTCH-mediated signaling is emerging as a potentially important pathway in controlling mammalian spermatogenesis (Garcia and Hofmann, 2013). In the present study, *DLK1* was up-regulated ~3-fold by gonadotrophin stimulation. This gene encodes a NOTCH ligand that hitherto has been recognized to promote differentiation in non-testicular tissues (Gubina *et al.*, 1999; Raghunandan *et al.*, 2008; Waddell *et al.*, 2010). *MYC*, the gene that encodes the V-myc avian myelocytomatosis viral oncogene homolog, a downstream target of many signaling pathways including that of NOTCH, was induced. Other targets of NOTCH include members of the HES and HES-related families of bHLH transcription factors (Bray and Bernard, 2010), and in the present study *HES7* (hes family bHLH transcription factor 7) and *HEYL* (HES-related family bHLH transcription factor with YRPW motif-like) were up-regulated. *HEYL* has been noted to promote differentiation of neural progenitor cells (Mukhopadhyay *et al.*, 2009; Jalali *et al.*, 2011), and in one study (Mukhopadhyay *et al.*, 2009), expression of *HEYL* was inversely related to that of *HEY1* (hes related family BHLH transcription factor with YRPW motif 1). The latter finding is of interest because this reciprocal relationship in expression of *HEYL* and *HEY1* was recapitulated in the juvenile monkey testis after 96 h of gonadotrophin stimulation.

Robust changes in a large variety of genes that have known functions in cell adhesion and structural plasticity were observed following

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gonadotrophin stimulation of the juvenile monkey testis. These included genes encoding actins, actin binding and remodeling proteins as well as tubulins that may be associated with the initial formation of the intracellular structures in SCs that are required to anchor the blood-testis barrier and to develop connections to germ cells (Lie *et al.*, 2010). Consistent with this idea was the increase in expression of the gene encoding CLDN11, a major component of the blood-testis barrier.

Further evidence for remodeling of extra-cellular matrix is suggested by the findings that *NTN4* (Netrin 4), which encodes a laminin-related protein, was down-regulated whereas the genes encoding annexin A1 (ANXA1) and annexin A2 (ANXA2), calcium and phospholipid binding proteins, were up-regulated. These extra-cellular matrix proteins have been implicated in the regulation of stem cell fate in other systems (Jung *et al.*, 2007; Staquicini *et al.*, 2009; Bizzarro *et al.*, 2010) and therefore may have previously unrecognized analogous roles in the testis. Similarly, *SPP1* that encodes secreted phospoprotein 1; an extra-cellular matrix or cell surface protein that also may have intracellular activities (Denhardt and Guo, 1993) was transiently up-regulated at 48 h. SPP1 promotes differentiation of osteoblasts (Denhardt and Guo, 1993) and is expressed in undifferentiated mouse spermatogonia as well as in hematopoietic stem cells and the hair follicle stem cell niche (Mizukami *et al.*, 2008).

Other genes also known to promote differentiation in non-testicular tissues were up-regulated by gonadotrophin stimulation in the present study. Examples include *NME2* (nucleoside diphosphate kinase 2) and *NDRG2* (N-myc downstream regulated gene 2) genes that promote differentiation of embryonic stem (ES) cells (Zhu *et al.*, 2009) and olfactory bulb stem cells (Nieto-Estevez *et al.*, 2013). Similarly, *NACC1* (nucleus accumbens associated 1), a master regulator of pluripotency that initiates the differentiation of ES cells (Kim *et al.*, 2008), was up-regulated by LH and FSH stimulation.

Several genes that play a role in innate immunity or inflammation were regulated by gonadotrophin treatment indicating a significant change in the immune environment of the testis. The most interesting of these to be up-regulated were *CCL2* and *CX3CR1*. *CCL2* encodes a member of the C-C subfamily of chemokines (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1). In the rodent, CCL2 has been implicated as a critical stimulus for the recruitment of monocytes into testis that, in primates, plays a major role in regulating the pubertal expansion of the resident macrophage population in this tissue (Raburn *et al.*, 1993; Winnall and Hedger, 2013). *CX3CR1* is expressed by macrophages and encodes the receptor for fractalkine (Combadiere *et al.*, 1998), which is considered to be a key regulator of recruitment of this cell type to the testis (Winnall and Hedger, 2013 and Hedger, unpublished observation). That activity or size of the population of testicular macrophages in the juvenile monkey was increased by gonadotrophin stimulation was further suggested by the finding that several genes known to be expressed by these immune cells were up-regulated by LH and FSH treatment. In this regard, macrophages have been implicated in governing the spermatogonial niche (DeFalco *et al.*, 2014), which is recognized to contribute to regulating the balance between differentiation and renewal of these cells (Valli *et al.*, 2015).

In summary, using the juvenile male rhesus monkey we have developed a primate model to investigate the genomic and metabolic underpinnings that are responsible for the decision by undifferentiated type A spermatogonia to commit to a path of differentiation. Our results indicate that in the monkey, and likely other higher primates including man, the early trigger of spermatogonial differentiation at puberty is the activation of a complex gonadotrophin-dependent network of families of testicular genes. This gene network is posited to govern paracrine/juxtacrine signaling from somatic cells within the testis and structural changes underlying spermatogonial niche formation that together are temporally linked to the down-regulation of spermatogonial genes that maintain stemness thereby resulting in a genetic landscape favoring differentiation. The key genes in regard to the latter appear to be *GFRA1, NANOS2* and *ZBTB16*, although the cell biology underlying their down-regulation remains an intriguing mystery. In contrast, genes traditionally recognized to be critical for spermatogonial differentiation in rodents, such as *KIT* and *SOHLH1* and *SOHLH2*, were not induced over the 96 h of gonadotrophin stimulation, and the commitment of undifferentiated spermatogonia to a pathway of differentiation was brought into play in the absence of evidence of robust activation of RA signaling. The initiation of spermatogonial differentiation, however, was associated with the apparent recruitment of macrophages to the testis. Finally, and perhaps most importantly, our results suggest, first, that functions of genes established to govern spermatogonial differentiation in the mouse may not necessarily translate directly to the primate testis, and, second, that several genes hitherto unrecognized as regulators of spermatogonia cell fate may now need to be considered in this regard.

Supplementary data

Supplementary information are available at *Human Reproduction* online.

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Authors' roles

S.R.: Contributed to experimental design, supervised the maintainance of chronically catheterized monkeys in remote infusion cages, conducted surgical procedures, performed hormone assays, responsible for immunohistofluorescence, prepared figures, contributed to data analysis and writing manuscript. W.H.W.: Responsible for pathway analysis, overall mining of the RNA-Seq data, supervising RT-qPCR analyses and writing the manuscript. P.A.: Played a major role in describing the testicular transcriptome and conducting pathway analysis, and contributed to data analysis and writing manuscript. R.S.: Conducted the pipepline analysis of the RNA-Seq data generated by the Illumina platform. G.R.M.: Responsible for all aspects of morphometric analysis of Bouins fixed testicular tissue. A.S.: Assisted WHW with bio-informatics and performed RT-qPCR assays. S.N.: Conducted RT-qPCR analyses. A.B.: Provided support and funding for PA, contributed to discussion of results, read and edited manuscript. U.R.C.: Directed the pipepline analysis of the RNA-Seq data generated by the Illumina platform, and contributed to developing strategies for sequence analysis and data mining. M.P.H.: Responsible for

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mining RNA-Seq data with respect to immune genes and contributed to the preparation of the manuscript. T.M.P.: Responsible for overall experimental design, obtaining financial support to conduct the study, and guaranteeing that the experiments were conducted in accord with NIH and University of Pittsburgh guidelines for the care and treatment of experimental animals. Assisted with all surgical procedures. Guided strategy for data analysis and presentation. Principal writer of the manuscript and provided final approval for submission of the paper.

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Conflict of interest

None declared.

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