

# Supplementary Materials for

## **Towards a “Testis in a Dish”: Generation of Mouse Testicular Organoids that Recapitulate Testis Structure and Expression profiles**

Aviya Stopel *et al.*

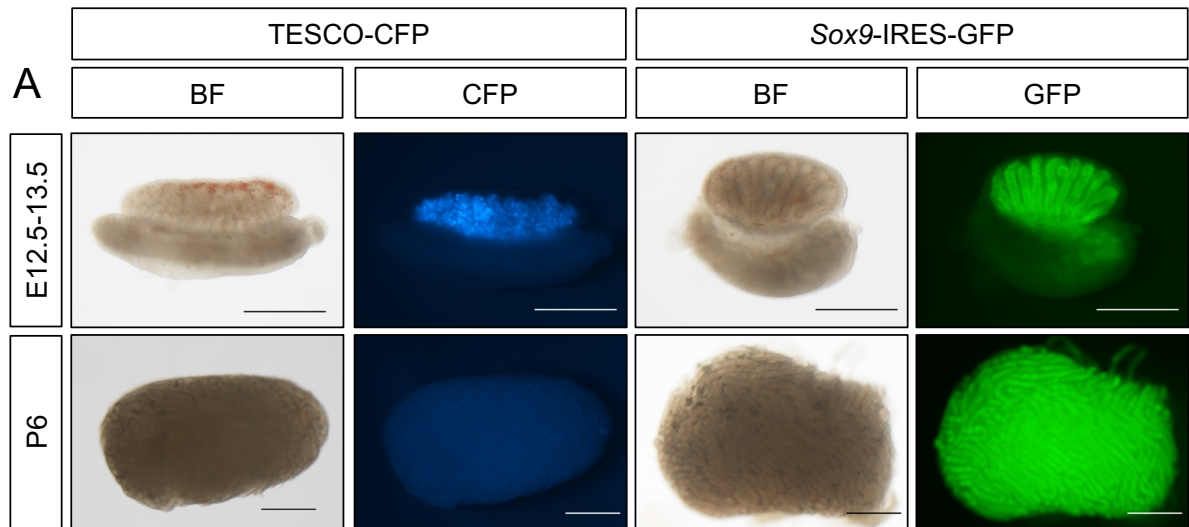
\*Corresponding author. Email: [Nitzan.Gonen@biu.ac.il](mailto:Nitzan.Gonen@biu.ac.il)

### **This PDF file includes:**

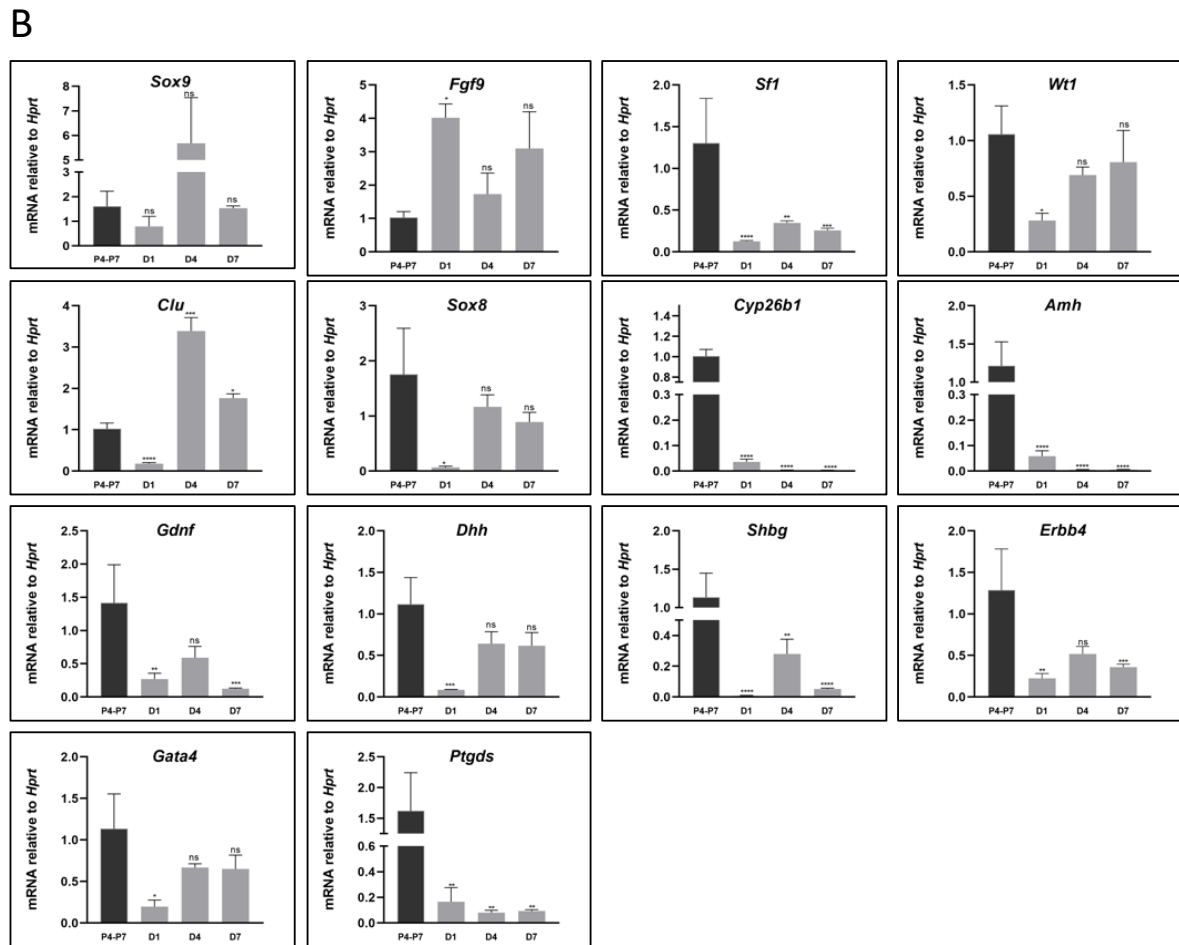
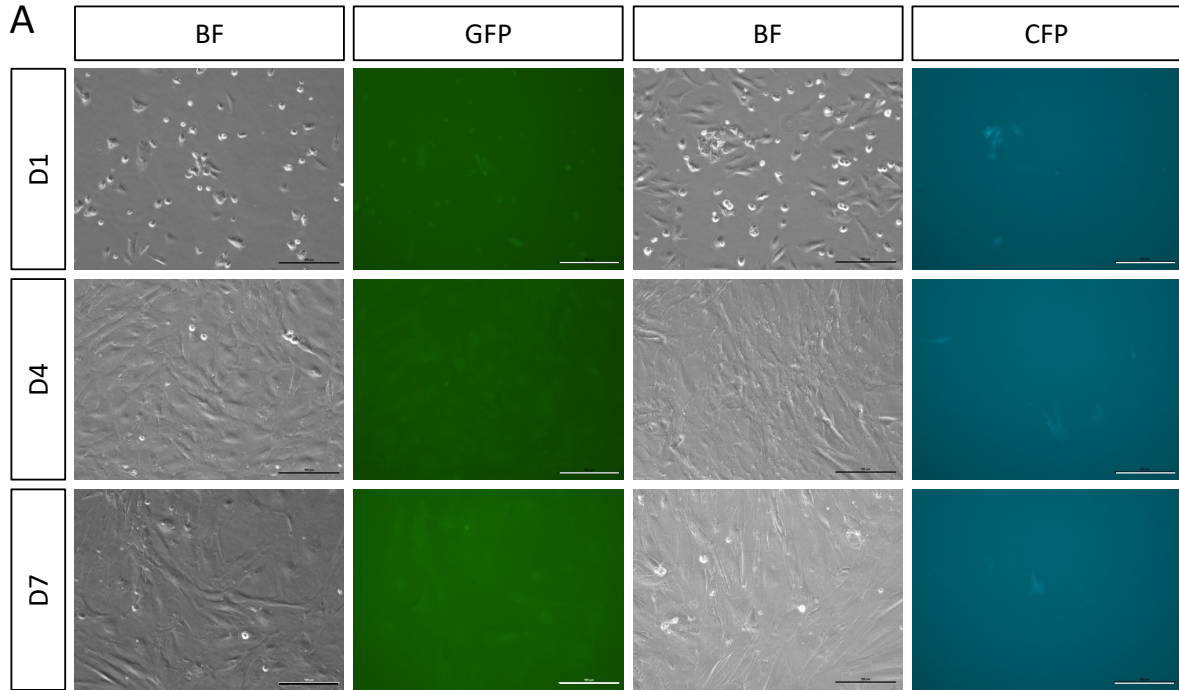
Figs. S1 to S10  
Tables S1 to S4

### **Other Supplementary Materials for this manuscript include the following:**

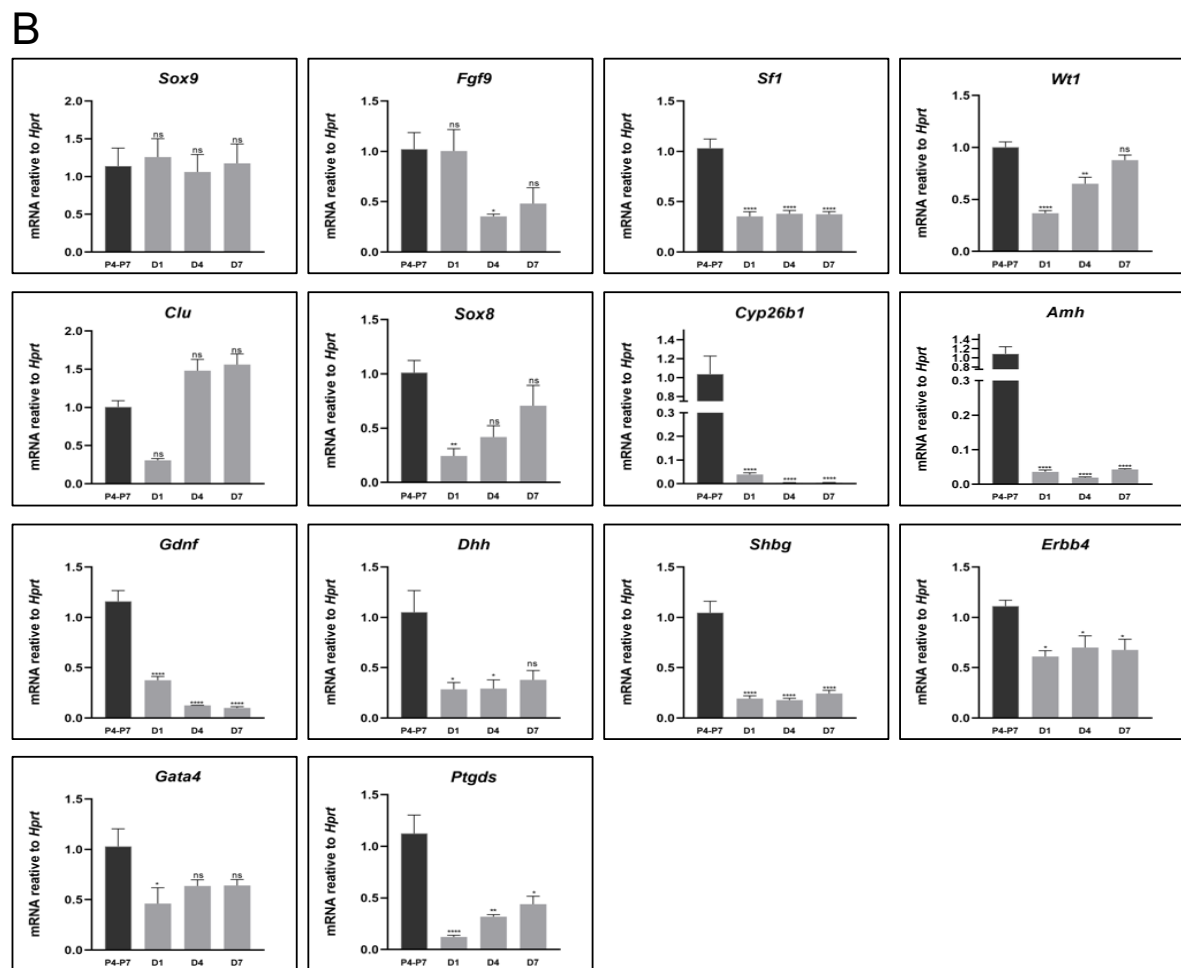
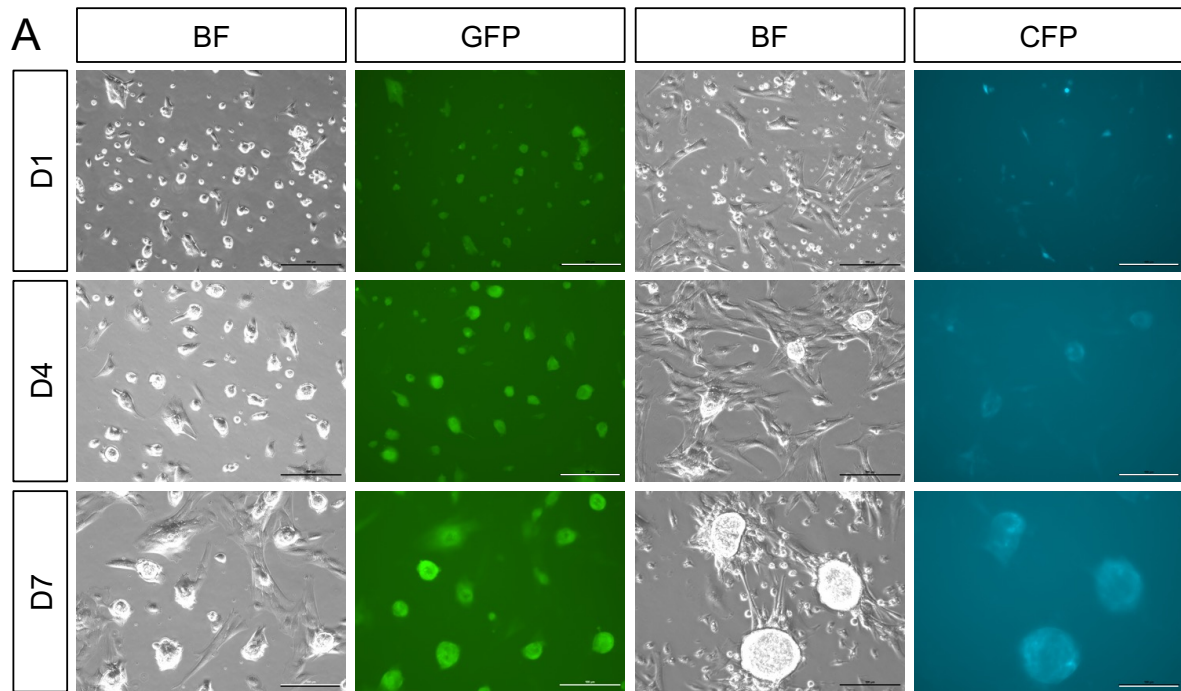
Movies S1 to S6



**Supplementary Figure 1. Sertoli cell-specific reporter mouse lines used for testicular harvest.** Bright-field (BF) and fluorescent images of testes from either E12.5-13.5 embryos or P6 pups harvested from *Sox9*-IRES-GFP or TESCO-CFP mice. Scale bars, 500  $\mu$ m.

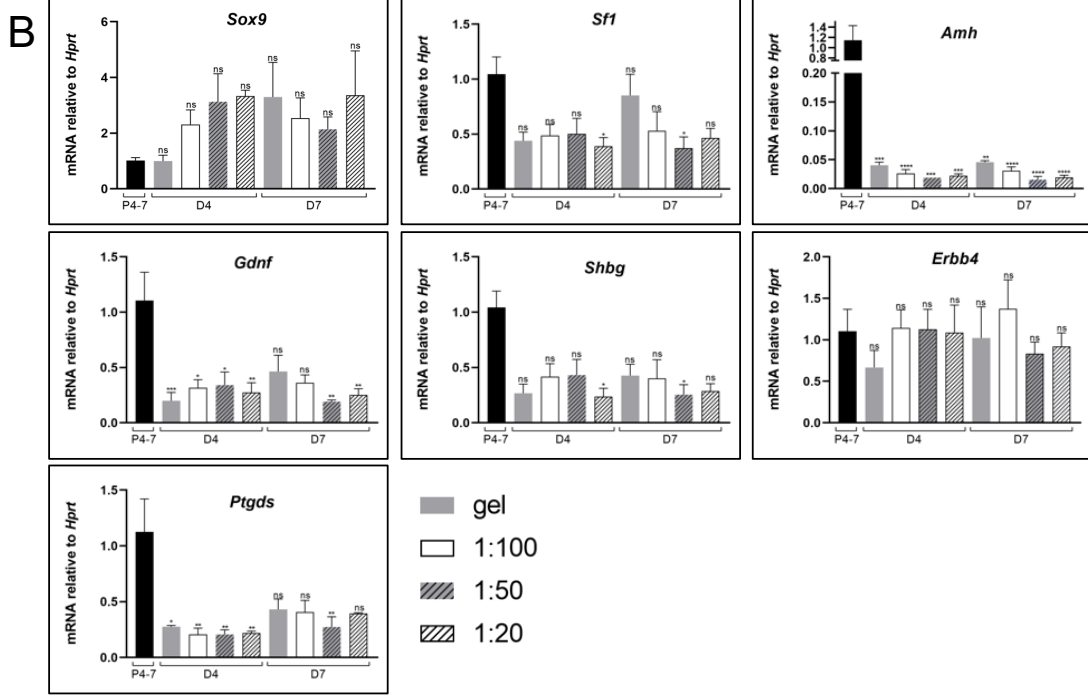
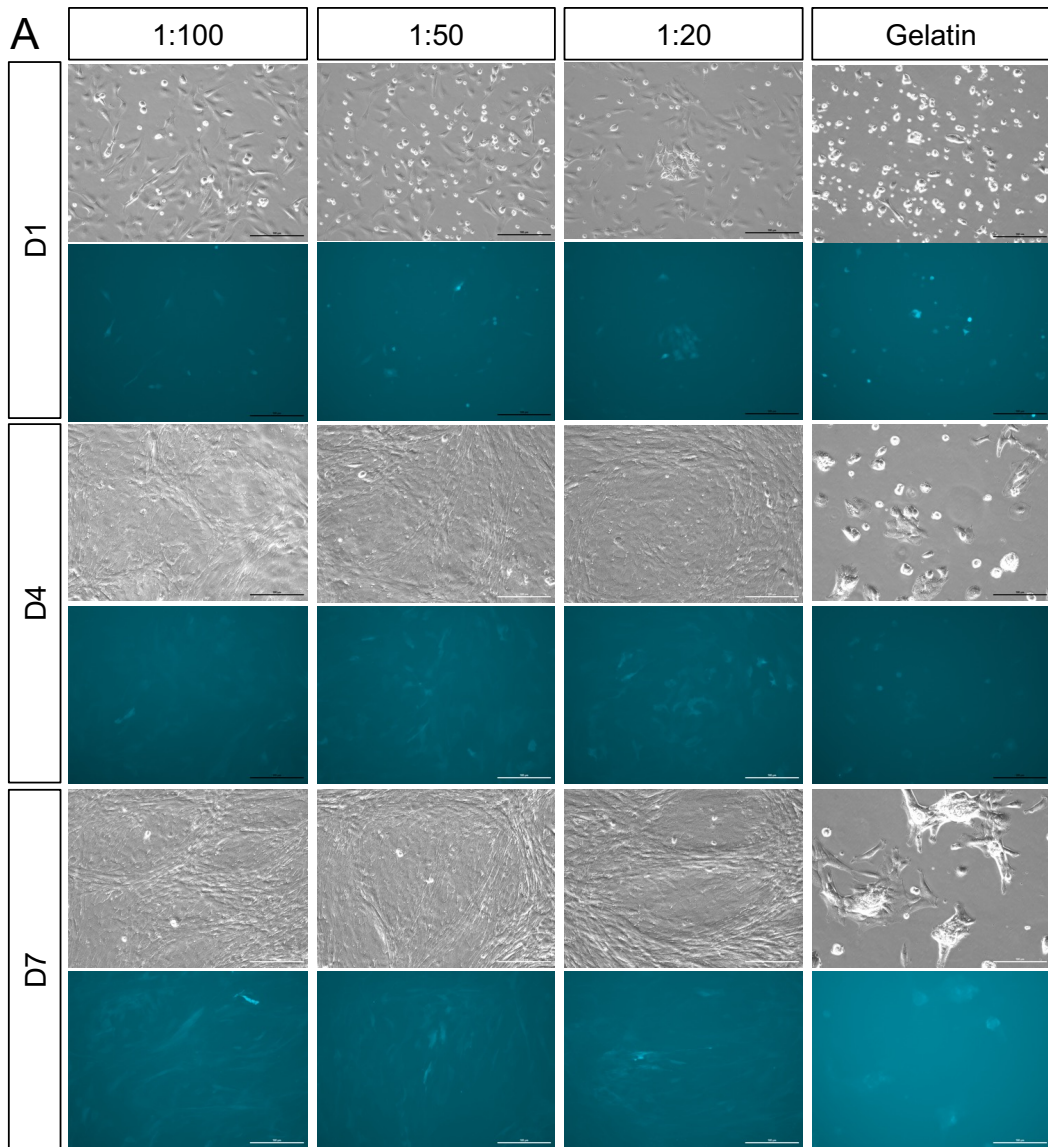


**Supplementary Figure 2. Testicular cells cultured in 2D with serum-based media.** (A) BF and fluorescent images of testicular cells harvested from either *Sox9*-IRES-GFP or TESCO-CFP pups. Cells were cultured for 7 days on 0.2% gelatin-coated dishes in Ad-DMEM/F12 media supplemented with 2% FBS. Scale bars, 100  $\mu$ m. (B) Quantitative PCR (qPCR) analysis of testicular cells from TESCO-CFP mice, cultured for 1-7 days in serum-based media. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns - not significant.  $N=3$ .



**Supplementary Figure 3. Testicular cells cultured in 2D with testis-defined media. (A)** BF and fluorescent images of testicular cells harvested from either *Sox9*-IRES-GFP or TESCO-

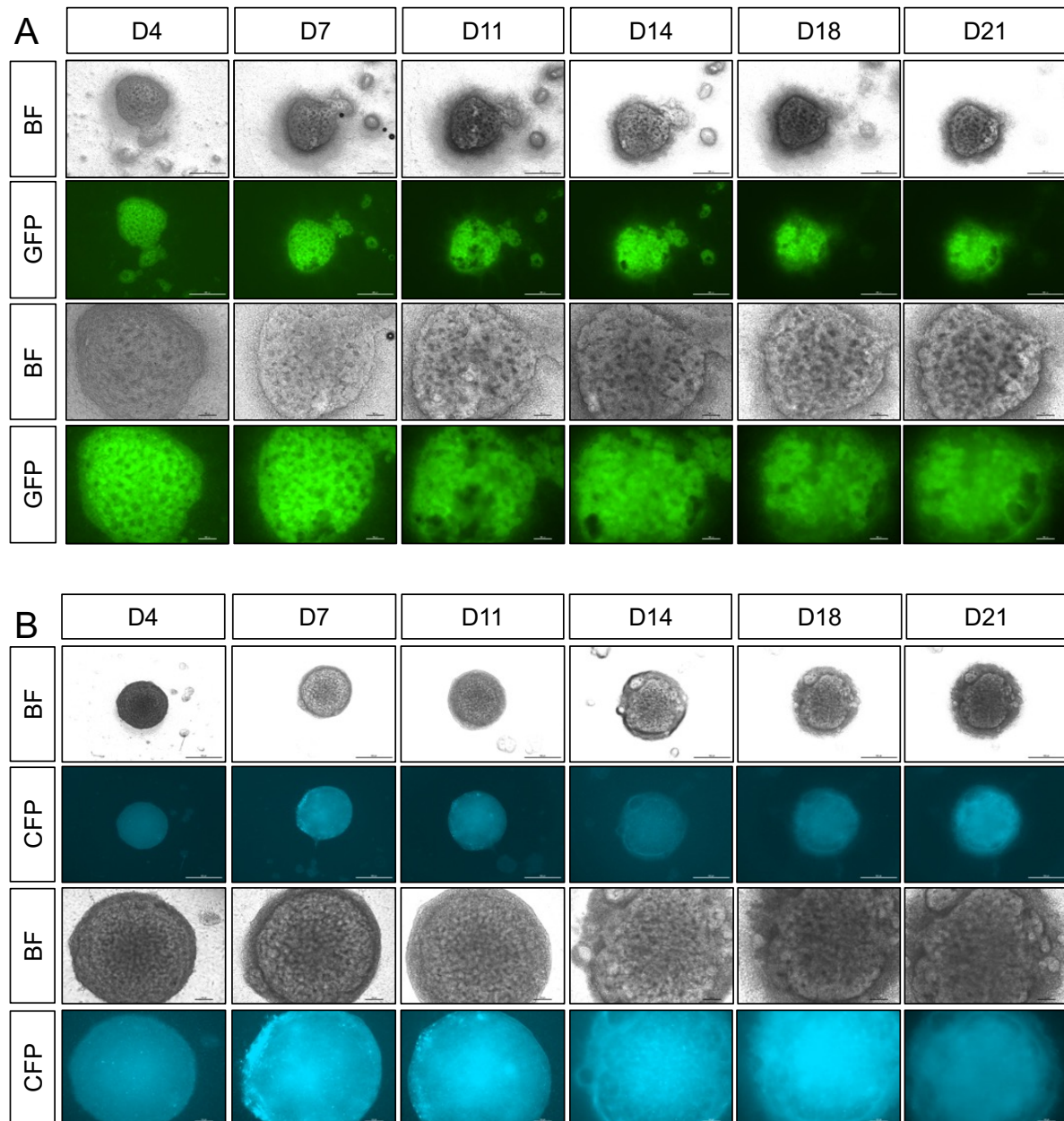
CFP pups. Cells were cultured for 7 days on 0.2% gelatin-coated dishes in defined media containing Ad-DMEM/F12 media supplemented with testicular growth factors as detailed in Supplementary Table 1. Scale bars, 100  $\mu\text{m}$ . (B) qPCR analysis of testicular cells from TESCO-CFP mice, cultured for 1-7 days with defined media. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns - not significant.  $N=5$ .



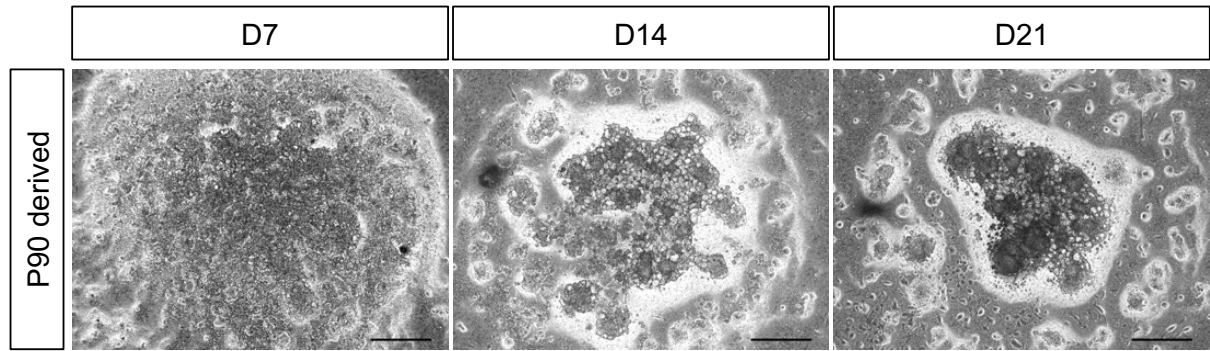
**Supplementary Figure 4. Testicular cells cultured on Geltrex coating in defined media.**

(A) BF and fluorescent images of testicular cells harvested from TESCO-CFP pups and cultured on various Geltrex dilutions (1:100, 1:50 and 1:20) or 0.2% gelatin (gel) coated wells. Scale bars, 100  $\mu\text{m}$ . (B) qPCR analysis of testicular cells from TESCO-CFP mice, cultured for 1-7 days on defined media and either Geltrex or Gelatin coating. Data are presented as mean  $2^{-\Delta\Delta\text{Ct}}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns - not significant.  $N=5$ .



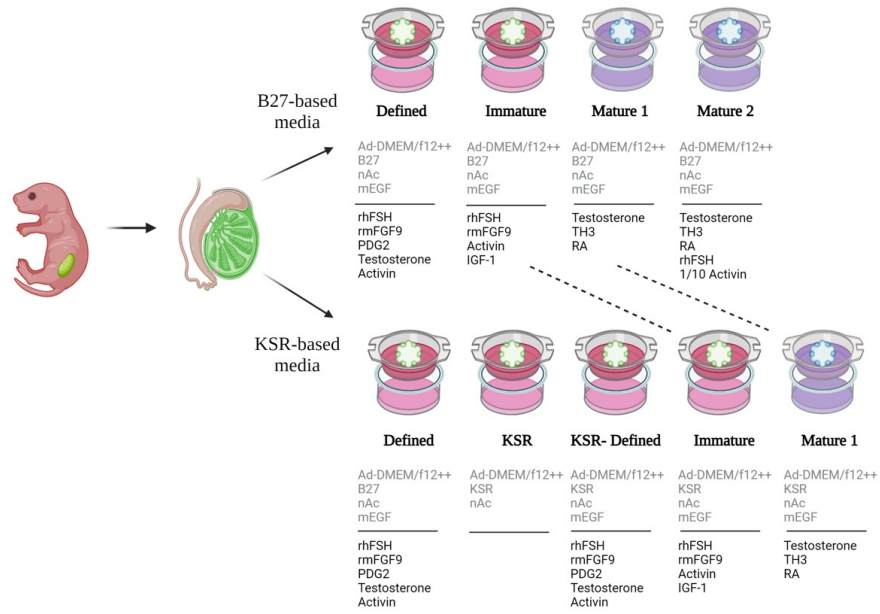


**Supplementary Figure 5. Testicular neonatal organoids cultured on transwell inserts.** (A) Representative BF and fluorescent images of organoids derived from *Sox9*-IRES-GFP testicular cells and cultured for up to 21 days on transwell inserts. (B) Representative BF and fluorescent images of organoids derived from TESCO-CFP testicular cells and cultured for up to 21 days on transwell inserts. Scale bars, 100  $\mu$ m.

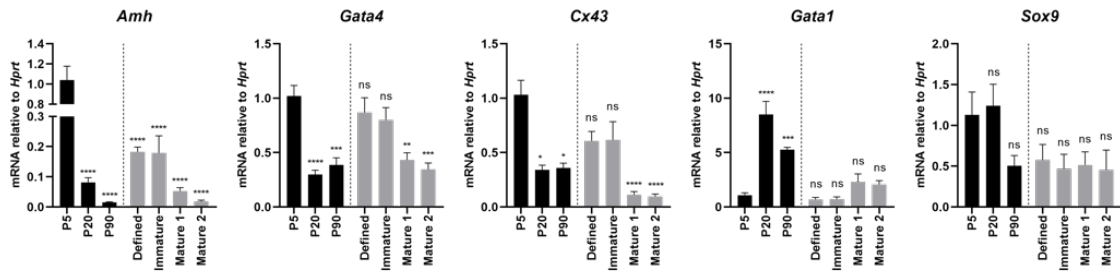


**Supplementary Figure 6. Organoids generated from mature testes.** Representative BF images of organoids generated from P90 primary testicular cells, cultured for up to 21 days on transwell inserts in defined media. Scale bar, 500  $\mu\text{m}$ .

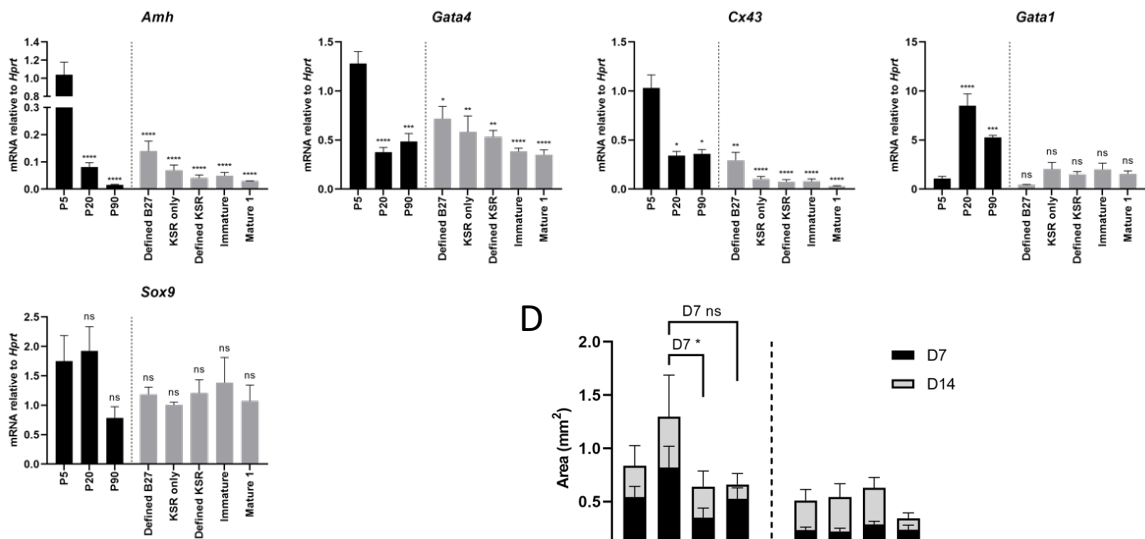
**A**



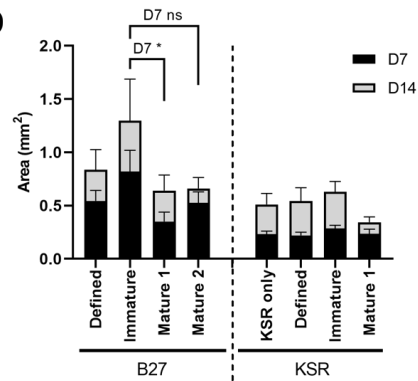
**B B27-based media**



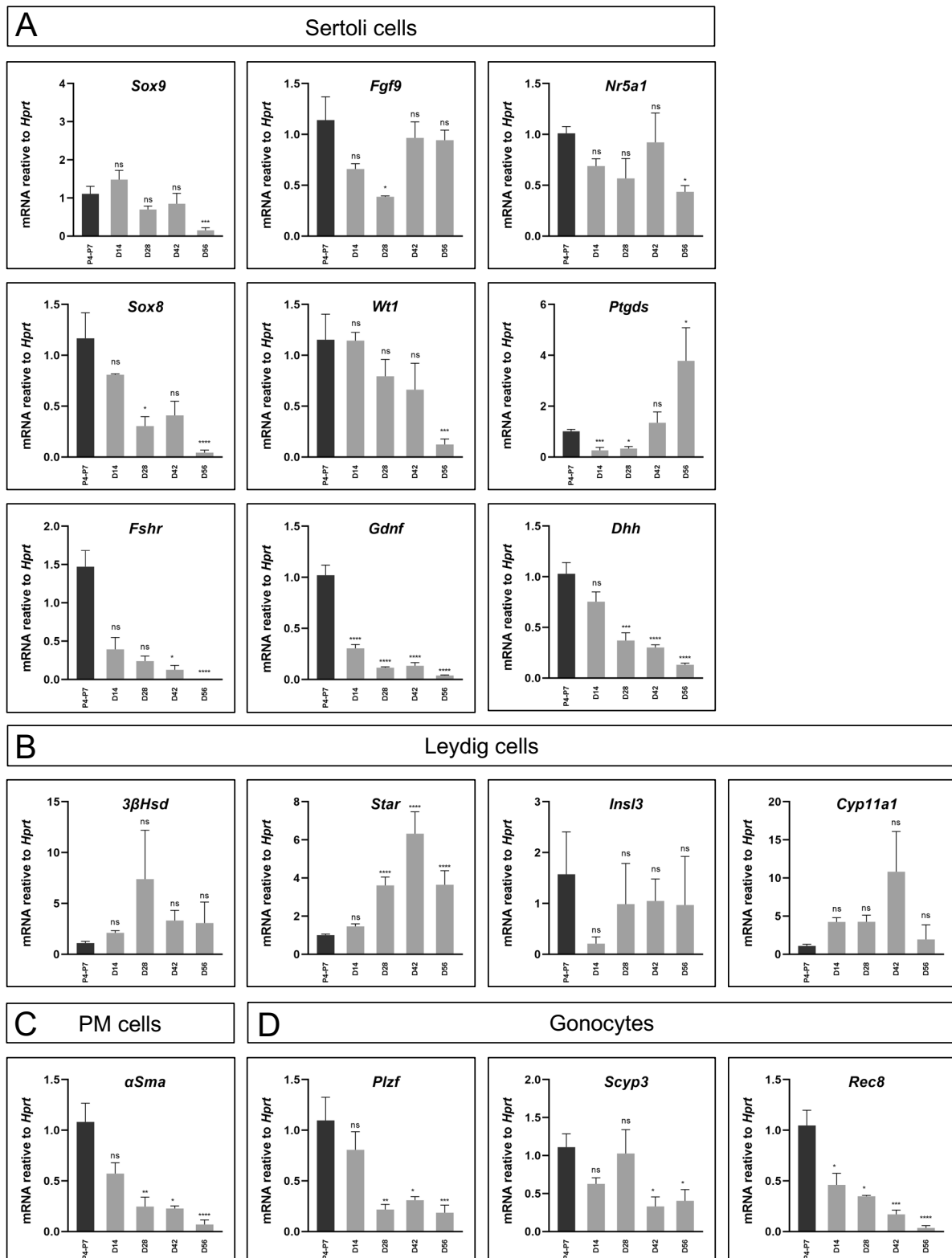
**C KSR-based media**



**D**

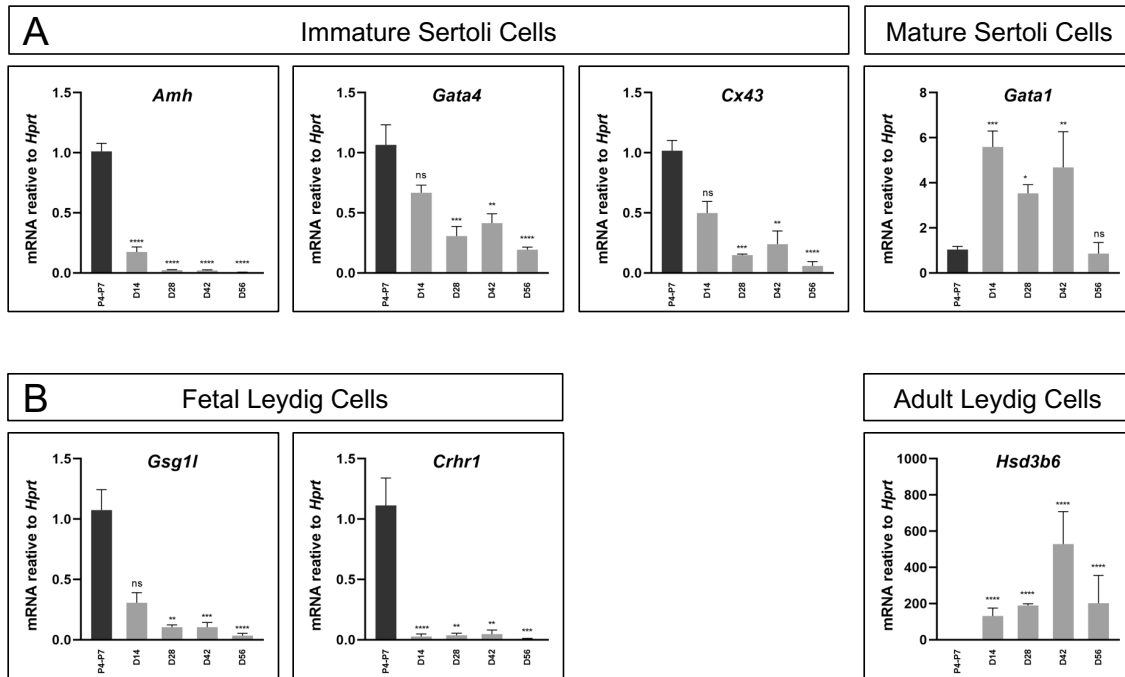


**Supplementary Figure 7. Refinement of media composition for immature and mature state of testicular organoids.** (A) Schematic representation of the surveyed media compositions on either B27-based or KSR-based media. The invariable additions are labelled in gray and the growth factors added to each media are labelled in black. For full media composition see Supplementary Table 1. Scheme created using BioRender. (B) qPCR analysis of Sertoli cell markers in organoids cultured in B27-based media for 14 days, compared to P5, P20 and P90 *in vivo* testis. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns - not significant.  $N=4-5$ . (C) qPCR analysis of Sertoli cell markers in organoids cultured on KSR-based media for 14 days, compared to P5, P20 and P90 *in vivo* testis. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns - not significant.  $N=4-5$ . (D) Organoid area on days 7 and 14 in B27- and KSR-based media. D7 bars are in black, D14 bars are cumulative and shaded grey. Data are presented as mean area  $\pm$ SEM. Statistical significance is shown for mature 1 and mature 2 vs. immature media at day 7 of culture. \* $P < 0.05$ , ns- not significant.  $N=5-8$ .

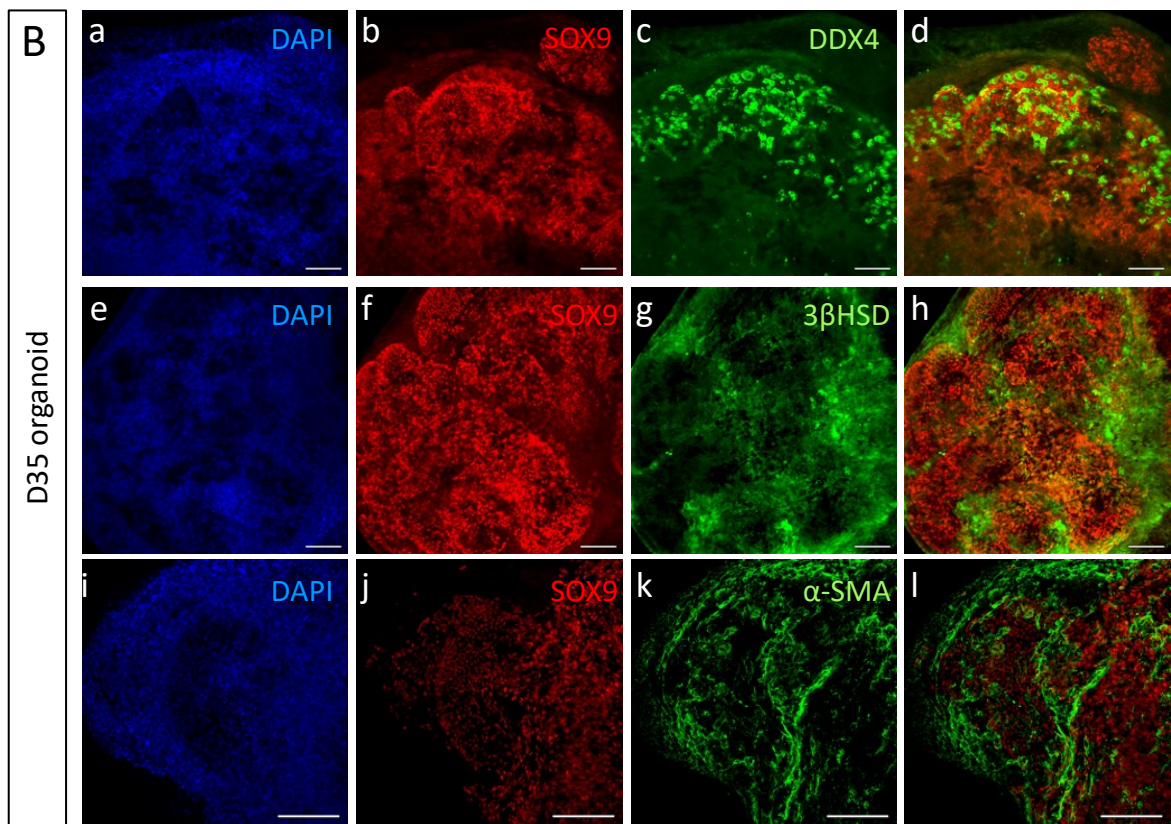
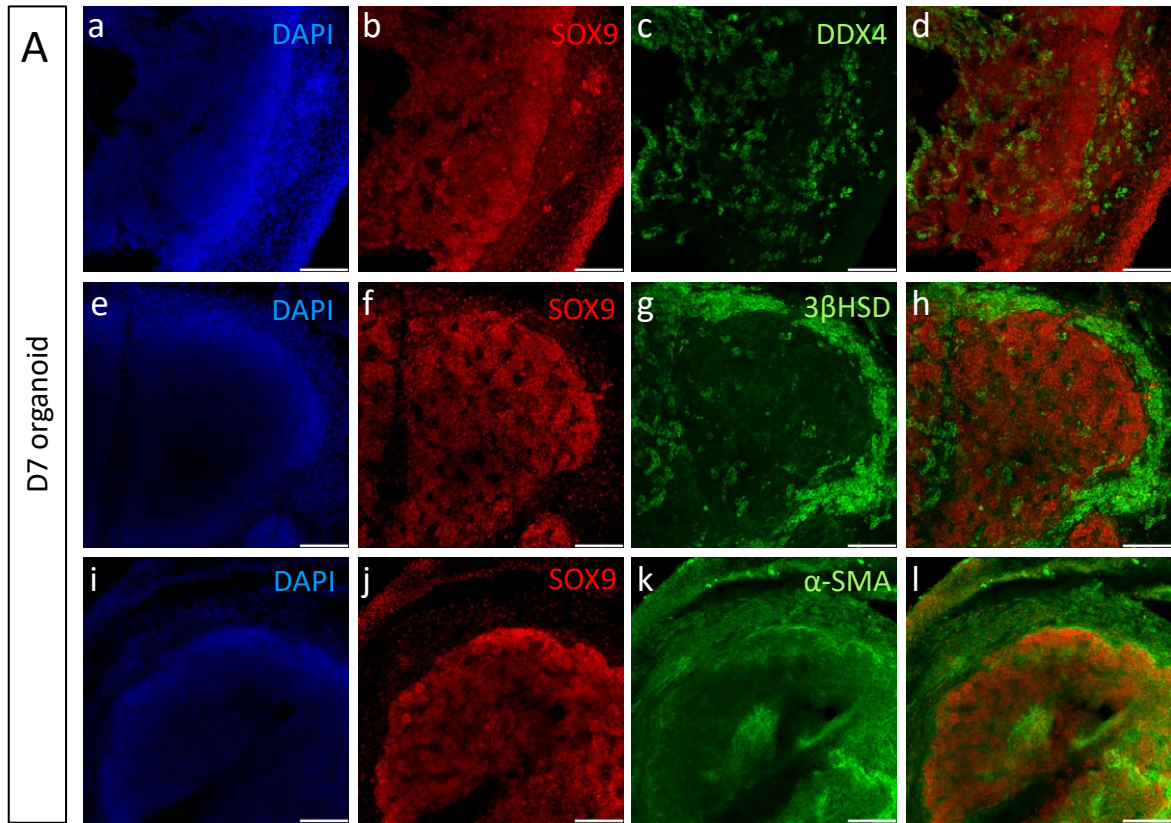


**Supplementary Figure 8. Gene expression profiles of organoids cultured in transition media over time.** (A-D) Quantitative RT-PCR was performed on mRNA extracted from neonatal testes, or TESCO-CFP organoids cultured for the indicated number of days on transition media assessing Sertoli cell markers (A), Leydig cell markers (B) or other testicular

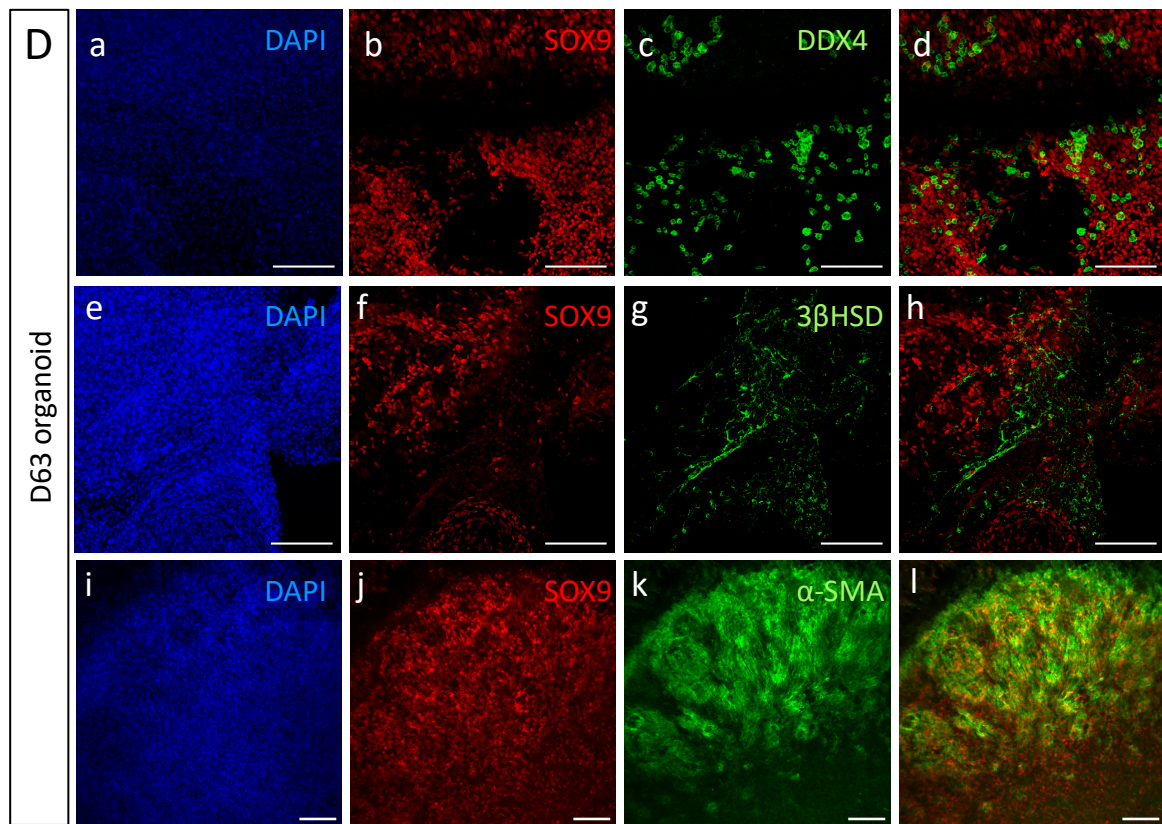
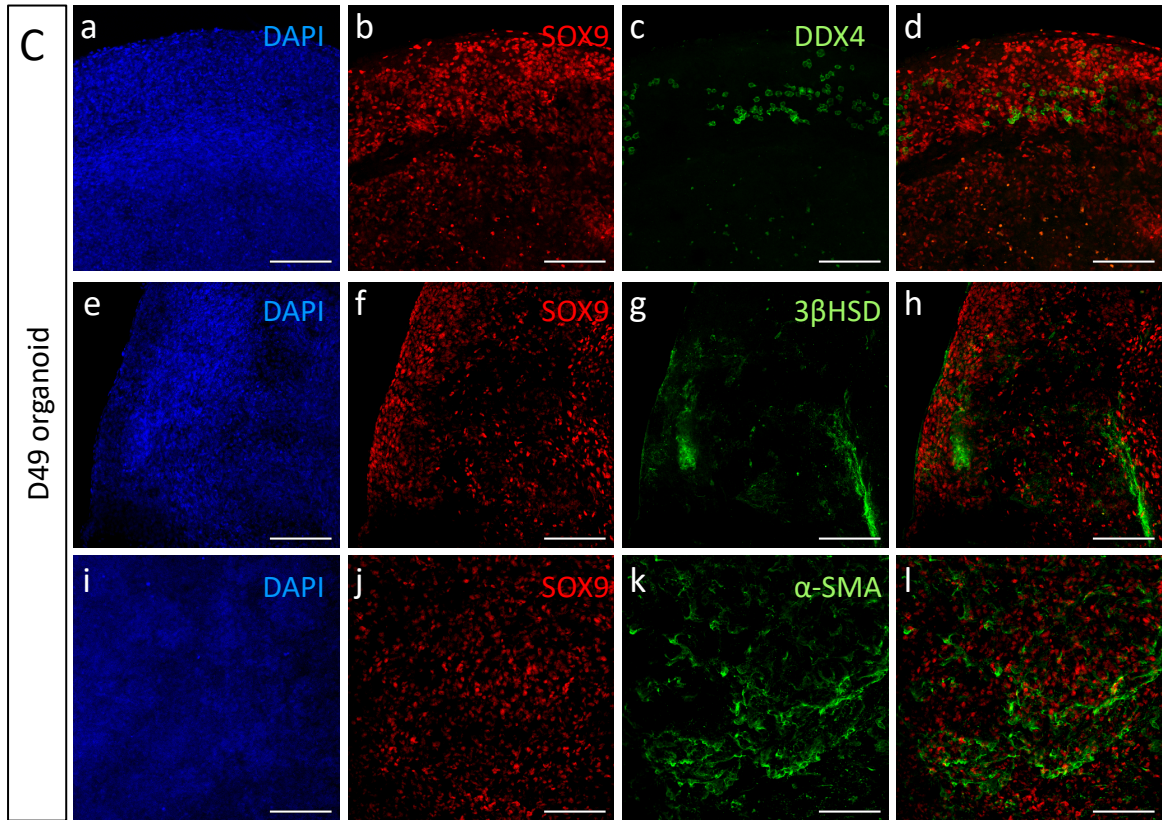
cells markers (C-D). Gene names are presented in the title. D denotes culture day of organoids, grey. P denotes days postpartum (dpp) of *in vivo* testis, black. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns- not significant.  $N=3-6$ .



**Supplementary Figure 9. Transition media promotes maturation of Sertoli and Leydig cells.** (A-B) Quantitative RT-PCR was performed on mRNA extracted from neonatal testes, or TESCO-CFP organoids cultured for the indicated number of days on transition media assessing Sertoli cell immature vs. mature markers (A), or fetal Leydig cell vs. adult Leydig cell markers (B). Gene names are presented in the title. D denotes culture day of organoids, grey. P denotes days postpartum (dpp) of *in vivo* testis, black. Data are presented as mean  $2^{-\Delta\Delta C_t}$  values  $\pm$ SEM normalized to the housekeeping gene *Hprt*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns- not significant.  $N=3-6$ .







**Supplementary Figure 10. Testicular organoids on transition media preserve testicular structures and gonadal cell types for up to 9 weeks.** (A-D) Whole mount co-immunostaining of markers for the major types of testicular cells in organoids cultured for 7 days (A), 35 days (B), 49 days (C) and 63 days (D). SOX9 marks Sertoli cells, DDX4 labels gonocytes, 3 $\beta$ HSD marks Leydig cells and  $\alpha$ -SMA labels PM cells. Images on the right are merged views of all channels. Scale bars, 100  $\mu$ m.

Table S1. Media composition					
Media	Component	Final concentration	Manufacturer	Cat. number	
Serum based medium	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020	
	Fetal Bovine Serum (FBS)	2%	Thermo Fisher Scientific	10270106	
Defined medium	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020	
	B-27 supplement	1X	Thermo Fisher Scientific	17504044	
	N-Acetyl-L-cysteine	1.25mM	Sigma	A9165	
	Mouse EGF	50ng/ml	Thermo Fisher Scientific	PMG8043	
	Recombinant human FSH	22ng/ml	R&D Systems	5925-FS	
	Recombinant mouse FGF9	50ng/ml	R&D Systems	7399-F9	
	Prostaglandin D2 (PDG2)	0.5µg/ml	Cayman	12010	
	Testosterone	1µM	Sigma	T1500	
	Recombinant Human/Mouse/Rat Activin A	50ng/ml	R&D Systems	338- AC	
	Immature	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010
		Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122
L-glutamine		2mM	Biological industries	03-020-1A	
B-27 supplement		1X	Thermo Fisher Scientific	17504044	
N-Acetyl-L-cysteine		1.25mM	Sigma	A9165	
Mouse EGF		50ng/ml	Thermo Fisher Scientific	PMG8043	
Recombinant human FSH		22ng/ml	R&D Systems	5925-FS	
Recombinant mouse FGF9		50µg/ml	R&D Systems	7399-F9	
Recombinant Human/Mouse/Rat Activin A		50ng/ml	R&D Systems	338- AC	
Recombinant human IGF-1		100ng/ml	R&D Systems	291-G1	
Mature1	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020-1A	
	B-27 supplement	1X	Thermo Fisher Scientific	17504044	
	N-Acetyl-L-cysteine	1.25mM	Sigma	A9165	
	Mouse EGF	50ng/ml	Thermo Fisher Scientific	PMG8043	
	Testosterone	1µM	Sigma	T1500	
	Thyroid hormone (T3)	100nM	Tocris	6666	
	Retinoic acid	1µM	Sigma	R2625	
	Mature2	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010
Penicillin-Streptomycin		1x	Thermo Fisher Scientific	15140122	
L-glutamine		2mM	Biological industries	03-020-1A	
B-27 supplement		1X	Thermo Fisher Scientific	17504044	
N-Acetyl-L-cysteine		1.25mM	Sigma	A9165	
Mouse EGF		50ng/ml	Thermo Fisher Scientific	PMG8043	
Recombinant human FSH		22ng/ml	R&D Systems	5925-FS	
Testosterone		1µM	Sigma	T1500	
Thyroid hormone (T3)		100nM	Tocris	6666	
Retinoic acid		1µM	Sigma	R2625	
Recombinant Human/Mouse/Rat Activin A	5ng/ml	R&D Systems	338- AC		
KSR	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020-1A	
	KnockOut Serum Replacement (KSR)	10%	Thermo Fisher Scientific	10828028	
	N-Acetyl-L-cysteine	1.25mM	Sigma	A9165	
	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020-1A	
	KnockOut Serum Replacement (KSR)	10%	Thermo Fisher Scientific	10828028	
	N-Acetyl-L-cysteine	1.25mM	Sigma	A9165	
	Mouse EGF	50ng/ml	Thermo Fisher Scientific	PMG8043	
	Recombinant human FSH	22ng/ml	R&D Systems	5925-FS	
	Recombinant mouse FGF9	50µg/ml	R&D Systems	7399-F9	
	Prostaglandin D2 (PDG2)	0.5µg/ml	Cayman	12010	
	Testosterone	1µM	Sigma	T1500	
Recombinant Human/Mouse/Rat Activin A	50ng/ml	R&D Systems	338- AC		
KSR - Defined	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010	
	Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122	
	L-glutamine	2mM	Biological industries	03-020-1A	
	KnockOut Serum Replacement (KSR)	10%	Thermo Fisher Scientific	10828028	
	N-Acetyl-L-cysteine	1.25mM	Sigma	A9165	
	Mouse EGF	50ng/ml	Thermo Fisher Scientific	PMG8043	
	Recombinant human FSH	22ng/ml	R&D Systems	5925-FS	
	Recombinant mouse FGF9	50µg/ml	R&D Systems	7399-F9	
	Prostaglandin D2 (PDG2)	0.5µg/ml	Cayman	12010	
	Testosterone	1µM	Sigma	T1500	
	Recombinant Human/Mouse/Rat Activin A	50ng/ml	R&D Systems	338- AC	
	Immature	Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010
		Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122
		L-glutamine	2mM	Biological industries	03-020-1A
		KnockOut Serum Replacement (KSR)	10%	Thermo Fisher Scientific	10828028
N-Acetyl-L-cysteine		1.25mM	Sigma	A9165	
Mouse EGF		50ng/ml	Thermo Fisher Scientific	PMG8043	
Recombinant human FSH		22ng/ml	R&D Systems	5925-FS	
Recombinant mouse FGF9		50µg/ml	R&D Systems	7399-F9	
Recombinant Human/Mouse/Rat Activin A		50ng/ml	R&D Systems	338- AC	
Recombinant human IGF-1		100ng/ml	R&D Systems	291-G1	
Mature1		Ad-DMEM-F12	/	Thermo Fisher Scientific	12634010
		Penicillin-Streptomycin	1x	Thermo Fisher Scientific	15140122
		L-glutamine	2mM	Biological industries	03-020-1A
		KnockOut Serum Replacement (KSR)	10%	Thermo Fisher Scientific	10828028
		N-Acetyl-L-cysteine	1.25mM	Sigma	A9165
	Mouse EGF	50ng/ml	Thermo Fisher Scientific	PMG8043	
	Testosterone	Sigma	T1500	T1500	
	Thyroid hormone (T3)	Tocris	6666	6666	
	Retinoic acid	Sigma	R2625	R2625	

**Table S2. Primers for genotyping mice**

<b>Primer name</b>	<b>Description</b>	<b>Sequence 5' to 3'</b>	<b>Product size</b>
CFP-F	CFP	CGTGACCACCCTGACCTGG	327 bp product
CFP-R	CFP	GTGGCGGATCTTGAAGTTGG	
GFP-F	GFP	TGCAGTGCTTCAGCCGCTAC	462 bp product
GFP-R	GFP	CCAGCAGGACCATGTGATCC	

Table S3. Primers for Real-time quantitative RT-PCR			
Primer name	Gene	Marker of	Sequence 5' to 3'
m3βHsd_F	3βHsd	Leydig cells	CTCAGTTCTTAGGCTCAGCAATTAC
m3βHsd_R			CCAAAGGCAAGATATGATTTAGGA
mAcrosin_F	Acrosin	Mature sperm	TGTCCGTGGTTGCCAAGGATAACA
mAcrosin_R			AATCCGGGTACCTGCTTGTGAGTT
mAmh_F	Amh	Immature Sertoli cells	CCACGGTTAGCACCAAATAGC
mAmh_R			CACACAGAACCTCTGCCCTACTC
mAr_F	Ar	Sertoli cells	CTGGGAAGGGTCTACCCAC
mAr_R			GGTGCTATGTTAGCGGCCTC
mClu_F	Clu	Sertoli cells	TGAAGGGCCAGTGTGAAAAGT
mClu_R			TTGAACAGTCCACAGACAAGATCTC
mCrhr1_F	Crhr1	Fetal Leydig cells	CCAGGATCAGCAGTGTGAGA
mCrhr1_R			TGTTGTGGTGTGTAGCGG
mCx43_F	Cx43	Adult Sertoli cell	GGTCTGAGAGCCCGAACTCTCCT
mCx43_R			CCCATGTCTGGGCACCTCTCTTT
mCyp11a1_F	Cyp11a1	Leydig cells	CACCTTCTGGAGGGAGAGTGG
mCyp11a1_R			ATGCCTGGAAGAAAAGCCGA
mCyp26b1_F	Cyp26b1	Sertoli cells/ FLC	AAGGGCTCCATGGGATTC
mCyp26b1_R			ACGACTGGAAGCCGGAAC
mDhh_F	Dhh	Sertoli cells	CCGCAACCACATCCACGTA
mDhh_R			CGGACCGCCAGTGAGTTATC
mErbB4_F	ErbB4	Sertoli cells	CAGCGCTTCTCAGTCAGTGT
mErbB4_R			CTGCTGTTCCAGGTCAGAGA
mFgf9_F	Fgf9	Sertoli cells	TCTCCCCAACGGTACTATCCA
mFgf9_R			CGAAGCGGCTGTGGTCTTT
mFshr_F	Fshr	Sertoli cells	TTGCCTGATGATGTTTTCCA
mFshr_R			CTGGCCCTCAACTTCTTCAG
mGata1_F	Gata1	Adult Sertoli cell	TGGGGACCTCAGAACCCTTG
mGata1_R			GGCTGCATTGGGGAAAGTG
mGata4_F	Gata4	Sertoli cells	CCCCAATCTCGATATGTTTGATG
mGata4_R			TTGACACACTCTCTGCCTTCTGA
mGdnf_F	Gdnf	Sertoli cells	GGGTGCGTTTTAACTGCCATA
mGdnf_R			GCCCAAACCCAAGTCAGTGA
mGsg11_F	Gsg11	Fetal Leydig cells	GAGGAGCCGACGTTTCATAGA
mGsg11_R			AGCACTGACGGTTAGCTCT
mHprt_F	Hprt	HK gene	GCAGTACAGCCCCAAAATGG
mHprt_R			GGTCCTTTTACCAGCAAGCT
mHsd3b6_F	Hsd3b6	Adult Leydig cells	GGAGGAGATCAGGTCCTGG
mHsd3b6_R			CTAGGATGGTCTGCCTGGG
mInsl3_F	Insl3	Leydig cells	GCTGCTACTGATGCTCCTGG
mInsl3_R			CTAGACCCGCAGCTTCTCTC
mNr5a1_F	Nr5a1	Sertoli cells	CCTCGATGTGAAATTCCTGAACA
mNr5a1_R			TCCTGGGCGTCTTTTACG
mPlzf_F	Plzf	Spermatogonial stem cells (SSC)	CCAGTTGGAGACGACCTACA
mPlzf_R			GTGGCAGAGTTTGCCTCAA
mPtgds_F	Ptgds	Sertoli cells	GGCTCCTGGACACTACACT
mPtgds_R			CATAGTTGGCCTCCACCACT
mRec8_F	Rec8	Meiotic spermatids	GCCCTAGAAGGTGCTGTTGG
mRec8_R			GTGGGGTCACCTCAGTGAGTAGG
mScyp3_F	Scyp3	Meiotic spermatids	GCAGTCTAGAATTGTTTCAGAGCCAGA
mScyp3_R			TCCAAACTCTTTATGAACTGCTCGTG
mShbg_F	Shbg	Sertoli cells	CCAAAATCAGCAAACCCCAT
mShbg_R			CTGGATCCCAGGTTGAAAC
mSox8_F	Sox8	Sertoli cells	AGCGAGAAGAGGCCGTTTG
mSox8_R			TCAGTACCAGAGTCTGAGTCG
mSox9_F	Sox9	Sertoli cells	AAGAAAGACCACCCCGATTACA
mSox9_R			CAGCGCCTTGAAGATAGCATT
mStar_F	Star	Leydig cells	GACGTCGGAGCTCTCTGCTT
mStar_R			GCCTTCTGCATAGCCACTC
mWtl_F	Wtl	Sertoli cells	TTGAATGCATGACCTGGAATCA
mWtl_R			TTCCCTTTAAGGTAGCTCCTAGGTT
mαSma_F	αSma	Peritubular myoid cells (PMC)	CCCCTGAAGAGCATCCGACA
mαSma_R			TGGCGGGGACATTGAAGGT

<b>Table S4. Antibody list</b>				
<b>Protein</b>	<b>Cat. No.</b>	<b>Raised in</b>	<b>Company</b>	<b>Dilution</b>
SOX9	AB5535	Rabbit	Merck Millipore	1:5000
DDX4	ab27591	Mouse	Abcam	1:300
$\alpha$ SMA	A2547	Mouse	Sigma	1:200
$\beta$ HSD	sc-515120	Mouse	Santa Cruz	1:200
$\gamma$ H2AX	ab11174	Rabbit	Abcam	1:1000
TRA98	ab82527	Rat	Abcam	1:200
AMH	sc-6886	Goat	Santa Cruz	1:200
CLAUDIN-11	Zy-364500	Rabbit	Invitrogen	1:100
REC8	ab192241	Rabbit	Abcam	1:200

**Movie S1.** Supplementary movie 1\_D21 organoid\_SOX9\_DDX4 b\_d

**Movie S2.** Supplementary movie 2\_D21 organoid\_SOX9\_3bHSD f\_h

**Movie S3.** Supplementary movie 3\_D21 organoid\_SOX9\_SMA j\_l

**Movie S4.** Supplementary movie 4\_D21 organoid\_AMH\_CLD11\_DDX4 m\_p

**Movie S5.** Supplementary movie 5\_D21 organoid\_SOX9

**Movie S6.** Supplementary movie 6\_D21 organoid\_DAPI