

SUPPLEMENTARY MATERIALS

In vitro models for testicular steroidogenesis: Current status and future perspectives

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SUPPLEMENTARY TEXTS

Supplementary Text S1: Search strategy for identifying Leydig cells and other cell types used in *in vitro* studies of testicular steroidogenesis

PubMed query run (19.10.2022 & 11.11.2024): ("H295R" [tw] OR "NCI-H295R" [tw] OR "I-10" [tw] OR "B-1-A-2" [tw] OR "BLT-1" [tw] OR "I-10/DDPh" [tw] OR "I-10/DDPi" [tw] OR "LC-540" [tw] OR "LK17" [tw] OR "MA-10" [tw] OR "K9" [tw] OR "MLTC-1" [tw] OR "R2C" [tw] OR "TM3" [tw] OR "I 10" [tw] OR "LC540" [tw] OR "LC 540" [tw] OR "MA10" [tw] OR "mLTC-1" [tw] OR "R 2 C" [tw] OR "TM-3" [tw] OR "Primary Leydig" [tw] OR "Primary testicular cell" [tw] OR "Primary mice Leydig" [tw] OR "Primary human Leydig" [tw] OR "Isolated Leydig" [tw] OR "Isolated testicular cell" [tw] OR "Isolated mice Leydig" [tw] OR "Isolated human Leydig" [tw]) and ("cell" [tw] OR "cells" [tw] OR "line" [tw])

Supplementary Text S2: Search strategy for identifying stem cell-based *in vitro* models used to study testicular steroidogenesis

Searching strategy for stem cell-based *in vitro* models for testicular steroidogenesis involved two PubMed query runs (A & B). The results from both queries were initially compiled, and afterward, duplicates were removed. **PubMed query run A: (21.10.2022 & 11.11.2024):** (fibroblast [tw] OR "fibroblasts" [tw]) AND (("Testis"[Mesh] OR "Testis" [tw] OR "Testicle" [tw] OR "Testes" [tw] OR "Steroidogenic cells"[tw] OR "male gonadal cells"[tw] OR "Leydig"[tw] OR "Leydig Cells/metabolism"[Mesh] OR "Testicular cells"[tw] OR "Gonadal Lineage"[tw] OR "testicular"[tw] OR "male gonad"[tw]) AND ("Gonadal Steroid Hormones"[Mesh] OR "Gonadal Steroid Hormones"[tw] OR "Sex steroid hormones" [tw] OR "Sex hormone" [tw] OR "Sex hormones" [tw] OR "Steroid hormones"[tw] OR "Progesterone Congeners"[Mesh] OR "Pregnenolone"[Mesh] OR "Pregnenolone" [tw] OR "17-alpha-Hydroxypregnenolone"[Mesh] OR "17-Hydroxypregnenolone" [tw] OR "Progesterone"[Mesh] OR "Progesterone"[tw] OR "Testosterone Congeners"[Mesh] OR "Androgens" [tw] OR "Androgen" [tw] OR "Androstane-3,17-diol"[tw] OR "Androstenediol"[tw] OR "Androstenedione"[tw] OR "Androsterone"[tw] OR "Dihydrotestosterone"[tw] OR "Testosterone"[tw] OR "Dehydroepiandrosterone"[tw] OR "steroidogenesis" [tw] OR "Steroidogenic"[tw] OR "Spermatogenesis"[Mesh] OR "Spermatogenesis"[tw] OR "Hypogonadism"[Mesh] OR "Hypogonadism"[tw] OR "Infertility, Male"[Mesh] OR "Infertility, Male"[tw] OR "sex development"[tw] OR "male fertility"[tw]))

PubMed query run B: (21.10.2022 & 11.11.2024): ("Induced Pluripotent Stem Cells"[Mesh] OR "IPSC" [tw] OR "IPSCs" [tw] OR "induced pluripotent stem cell" [tw] OR "induced pluripotent stem cells" [tw] OR "hiPSC" [tw] OR "hiPSCs" [tw] OR "Adult stem cells" [Mesh] OR "Stem cells/metabolism" [Mesh] OR "Adult stem cells" [tw] OR "Stem cell" [tw] OR "Stem cells" [tw] OR "Fetal Stem Cells"[Mesh] OR "Fetal Stem Cells"[tw] OR "Embryonic Stem Cells"[Mesh] OR "Embryonic Stem Cells"[tw] OR "Human Embryonic Stem Cells"[Mesh] OR "Human Embryonic Stem Cells"[tw] OR "Mouse Embryonic Stem Cells"[Mesh] OR "Mouse Embryonic Stem Cells"[tw] OR "Mesenchymal Stem Cells"[Mesh] OR "Mesenchymal Stem Cells"[tw] OR "Marrow Stromal Cells" [tw] OR "Stem Leydig cells" [tw] OR "bone marrow mesenchymal cells"[tw]) AND (("Testis"[Mesh] OR "Testis" [tw] OR "Testicle" [tw] OR "Testes" [tw] OR "Steroidogenic cells"[tw] OR "male gonadal cells"[tw] OR "Leydig"[tw] OR "Leydig Cells/metabolism"[Mesh] OR "Testicular cells"[tw] OR "Gonadal Lineage"[tw] OR "testicular"[tw] OR "male gonad"[tw]) AND ("Gonadal Steroid Hormones"[Mesh] OR "Gonadal Steroid Hormones"[tw] OR "Sex steroid hormones" [tw] OR "Sex hormone" [tw] OR "Sex hormones" [tw] OR "Steroid hormones"[tw] OR "Progesterone Congeners"[Mesh] OR "Pregnenolone"[Mesh] OR "Pregnenolone" [tw] OR "17-alpha-Hydroxypregnenolone"[Mesh] OR "17-Hydroxypregnenolone" [tw] OR "Progesterone"[Mesh] OR "Progesterone"[tw] OR "Testosterone Congeners"[Mesh] OR "Androgens" [tw] OR "Androgen" [tw] OR "Androstane-3,17-diol"[tw] OR "Androstenediol"[tw] OR "Androstenedione"[tw] OR "Androsterone"[tw] OR "Dihydrotestosterone"[tw] OR "Testosterone"[tw] OR "Dehydroepiandrosterone"[tw] OR "steroidogenesis" [tw] OR "Steroidogenic"[tw] OR "Spermatogenesis"[Mesh] OR "Spermatogenesis"[tw] OR

"Hypogonadism"[Mesh] OR "Hypogonadism"[tw] OR "Infertility, Male"[Mesh] OR "Infertility, Male"[tw] OR "sex development"[tw] OR "male fertility"[tw])

SUPPLEMENTARY TABLES

Supplementary Table S1: Overview of keywords and key terms used to identify relevant studies from abstracts, including additional characterization in the context of testicular steroidogenesis.

Search Term	Keywords/ Key terms	Advanced strategy to overcome limitations
STIMULATION	hCG	human chorionic gonadotropin chorionic gonadotropin chorionic gonadotropin hCG CG gonadotrophin gonadotrophic hormone gonadotropin gonadotropic hormone ●hCG: This term also captures the name of the receptor hCGLHR. Receptors were filtered by comparing the terms for the receptor. If the term was found both as hCG and LHR, the abstract was manually searched. ●hCG usage: hCG may not always be used as a stimulant; it might be included for a basic introduction to a study. Therefore, the abstracts of some studies (too many to be searched manually) were checked manually. Rarely was hCG binding confirmed. Most studies used hCG as a stimulant of steroidogenesis.
	LH	lutein LH LH, LH. LH: (LH) LH) LH/ lutropin lutrophin ●LH: This term also captures the name of the receptor hCGLHR. Receptors were filtered by comparing the terms for the receptor. If the term was found both as LH and LHR, the abstract was manually searched. ●LH usage: LH may not always be used as a stimulant; it might be included for a basic introduction of a study. Therefore, the abstracts of all studies were checked manually and such studies were excluded.
	cAMP analog	8-bromo-cAMP (Bu)2cAMP Br-cAMP 8-bromo cyclic adenosine monophosphate Bt2cAMP dibutyl cAMP 8-br-cAMP 8Br-cAMP 8-bromo(Br)-cAMP 8-bromoadenosine 3',5'-cyclic monophosphate dibutyryl-cAMP dbcAMP 8-Bromo Cyclic Adenosine Monophosphate 8-bromoadenosine 3',5'-cyclic monophosphate Bt(2)cAMP [(Bu)(2)cAMP] (Bu)(2)cAMP db-cAMP dibutyryl cAMP dibutyryl [(Bu)(2)]cAMP 8bromo-cAMP ●Searched only for synthetic cAMP analogs used for stimulation. ●We could not do a brief search using just "cAMP" as it would have included introductions and measurements of cAMP levels. Consequently, we may have missed a few papers that only report cells being stimulated with cAMP. However, cAMP is quite unstable, which is why most studies use cAMP analogs to stimulate steroidogenesis.
	Hydroxy cholesterol	22(R)OH-cholesterol 22R-hydroxycholesterol hydroxycholesterol 25-hydroxycholesterol 22-hydroxycholesterol 22(R)-hydroxycholesterol 22(R)OH cholesterol 25-hydroxy cholesterol ●The basic search strategy was sufficient.

ENZYMES		25-OHC	
	Forskolin	forskolin colforsin Coleonol	●The basic search strategy was sufficient.
	CYP11A1	CYP11A1 CYP11 CYP11. CYP11, (CYP11) CYP11: P450SCC Cytochrome P450 11A1 Cytochrome P450(Scc) Cytochrome P450 Family 11 P450(scc) cholesterol side-chain cleavage enzyme P450 side-chain cleavage enzyme side-chain cleavage enzyme	●The basic search strategy was sufficient.
	CYP17A1	CYP17A1 CYP17 CYP17, CYP17. (CYP17) CYP17: P450C17 cytochrome P450 Family 17 Steroid 17-Alpha-Hydroxylase P450-17 α cytochrome P450 17 α -hydroxysteroid dehydrogenase P450c17	●The basic search strategy was sufficient.
	CYP19A1	CYP19A1 CYP19 CYP19, CYP19. (CYP19) CYP19: Aromatase P-450AROM ARO ARO, ARO. ARO) ARO((ARO) ARO: Cytochrome P450 Family 19 Cytochrome P450 19A1 P450arom	●The basic search strategy was sufficient.
	AKR1C3	HSD17B5 AKR1C3 Aldo-Keto Reductase Family 1 Member C3 3-Alpha-Hydroxysteroid Dehydrogenase 17-Beta-HSD 5	●The basic search strategy was sufficient.
	HSD17B3	HSD17 HSD17. HSD17, (HSD17) HSD17: HSD17B3 HSD17B1 SDR12C2 17-Beta-HSD 17 β -HSD Hydroxysteroid 17-Beta Dehydrogenase 3 Hydroxysteroid Dehydrogenase 3	●The basic search strategy was sufficient.

ENZYMES		17βHSD 17beta-HSD 17-βHSD 17betaHSD1 17beta-HSD1 HSD17β1	
	HSD3B	HSD3B2 3β-HSD SDR11E2 HSD3B1 HSDB3B Beta-HSD SDR11E1 HSDB3A 3 Beta-Hydroxysteroid Dehydrogenase 3 Beta-HSD Type II HSD3B HSD3B. HSD3B, (HSD3B) HSD3B: Hsd3β 3βHSD2 3β-HSD 3β-hydroxysteroid dehydrogenase 3β-HSD Hsd3β 3βHSD1 3betaHSD	●The basic search strategy was sufficient.
	SRD5A1	SRD5A1 Alpha-Reductase Steroid 5-Alpha-Reductase	●The basic search strategy was sufficient.
HORMONES	P5	pregnenolone pregnenolone. pregnenolone, pregnenolone: (pregnenolone) Pregnenolone Pregnenolone/	●Pregnenolone was mostly mentioned in the context of STAR function descriptions and introductions. Therefore, the abstracts of all relevant studies were checked manually, and only those that measured pregnenolone were counted.
	P4	progesterone progesterone, progesterone. (progesterone) progesterone: Progestin Progesterone Progesterone/	●After reviewing the abstracts of 75 articles, only 4 did not evaluate progesterone levels, and in 15 of them, progesterone was listed in the MeSH terms. We decided to disregard these. The abstracts of studies focusing on stem-cell-based and advanced models for testicular steroidogenesis were all checked.
	DHEA/ DHEA-S	DHEA dehydroepiandrosterone DHEA-S dehydroepiandrosterone sulfate DHEA sulfate androstenolone sulfate	●It would be complicated to distinguish between DHEA and DHEA-S in their full names; therefore, the two hormones were merged into one category.
	A4	Androstenedione 4-Androstene-3,17-dione Androst-4-ene-3,17-dione 4-Androstenedione	●The basic search strategy was sufficient.
	A5	Androstenediol 5-Androstenediol Androst-5-enediol	●The basic search strategy was sufficient.
	T	testosterone Testosteron	●Testosterone frequently appears in introductions and as a MeSH term without being mentioned in the abstract. Additionally, it also captures dihydrotestosterone. Therefore, the abstracts of relevant studies were checked manually.

	E1/E2/E3	estrogen estrone estradiol estriol	●Apart from measuring E1/E2/E3 levels as a response, these hormones are often used as substances of interest for which effects were detected. Additionally, estrogen is part of the “estrogen receptor.” Therefore, the abstracts of relevant studies were checked manually.
HORMONES	DHT	DHT 5- α -dihydrotestosterone Dihydrotestosterone 5 α -DHT stanolone androstanolone	●The basic search strategy was sufficient.
	17-OHP5	17-OH-pregnenolone 17 α -Hydroxypregnenolone 17-hydroxypregnenolone	●The basic search strategy was sufficient.
	17-OHP4	17-OH-progesterone 17 α -Hydroxyprogesterone 17 α -OHP 17-OH progesterone hydroxyprogesterone 17-OHP	●The basic search strategy was sufficient.
	ADIOL	Androstenediol 3 α -Androstenediol 5 α -androstane-3 α ,17 β -diol 3 β -Androstenediol 5 α -androstane-3 β ,17 β -diol 3 α -diol 5 α -Androstane-3 α , 17 β -diol 3 α -DIOL	●The basic search strategy was sufficient.
	AN	Androsterone 3 α -hydroxy-5 α -androstane-17-one Androsterone. Androsterone, Androsterone: Androsterone Androsterone/ (androsterone)	●The basic search strategy was sufficient.
	DHP	dihydroprogesterone DHP 5 α -Dihydroprogesterone 5 α -DHP allopregnanedione 5 α -pregnane-3,20-dione	●The basic search strategy was sufficient.
	5α-A	Androstenedione 5 α -androstenedione 5 α -androstane-3,17-dione	●The basic search strategy was sufficient.
CHOL transport	CHOL transport	cholesterol cholesterol. cholesterol, cholesterol: (cholesterol) cholesterol/ cholesterol cholesterol	●Finding studies that evaluated cholesterol transport in general was challenging. The issue was further complicated by the presence of the “Cholesterol Side-Chain Cleavage Enzyme.” Therefore, if the term “cholesterol” appeared in the abstract outside the context of the “Cholesterol Side-Chain Cleavage Enzyme,” the study was included. This approach may lead to an overestimation of results due to studies that investigated cholesterol for other reasons. All abstracts of studies focusing on stem-cell-based and advanced models for testicular steroidogenesis were reviewed.
	STAR	STAR STAR. STAR, STAR:	●The basic search strategy was sufficient.

		(STAR) STAR/ STAR Steroidogenic Acute Regulatory Protein STARD1	
	TSPO	TSPO Translocator Protein	•The basic search strategy was sufficient.
	Lipid droplets	lipid accumulation lipid droplet	•The basic search strategy was sufficient.

Supplementary Table S2: Number of studies using *in vitro* models for testicular steroidogenesis identified through general keyword-based searches (Search Strategy 1), categorized by term of interest.

	STIMULATION					ENZYMES							CHOLESTEROL TRANSPORT			
	hCG	cAMP analog	Hydroxy CHOL	Forskolin	LH	CYP11A1	CYP19A1	AKR1C3	HSD17B3	CYP17A1	HSD3B	SRD5A1	CHOL transport	STAR	TSPO	Lipid droplets
Primary	136	64	19	13	5	56	48	10	1	17	63	10	69	2	12	2
H295R	7	0	13	0	47	26	94	92	5	30	58	2	49	2	19	3
MA-10	139	37	116	30	26	68	21	2	0	2	29	5	158	15	129	8
TM3	13	8	1	0	0	34	19	4	0	19	29	1	47	1	8	2
MLTC1	43	10	11	6	7	28	11	0	0	4	16	1	32	0	16	4
R2C	1	3	3	1	4	7	3	18	0	2	6	0	15	0	4	1
LC-like	3	6	10	4	5	12	8	0	0	6	13	0	5	0	0	1

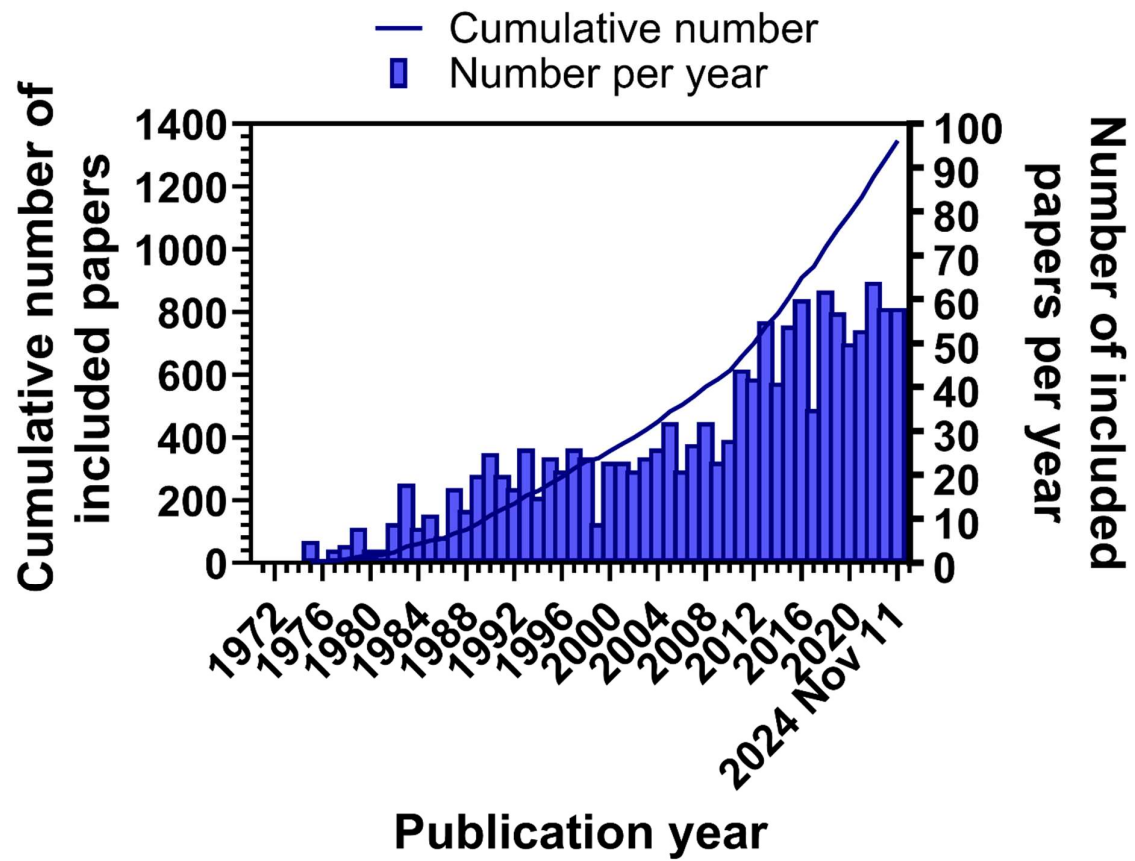
	HORMONE													
	P5	P4	DHEA/DHEA-S	A4	A5	T	E1/E2/E3	DHT	17-OHP5	17-OHP4	ADIOL	AN	DHP	5α-A
Primary	9	30	10	20	0	269	24	6	1	8	3	2	0	0
H295R	11	76	62	43	0	122	119	10	9	20	0	0	0	1
MA-10	17	180	1	5	0	30	3	5	0	2	0	0	3	0
TM3	0	7	2	1	0	117	5	0	0	0	0	0	0	0
MLTC1	3	46	1	4	0	29	0	1	0	2	0	0	0	0
R2C	2	14	0	1	0	11	5	0	0	0	0	0	0	0
LC-like	0	4	1	1	0	24	3	1	0	0	0	0	0	0

Supplementary Table S3: Number of studies using *in vitro* models for testicular steroidogenesis identified through targeted chemical searches (Search Strategy 2), categorized by term of interest and focused on chemical-specific effects on hormone levels during male steroidogenic processes.

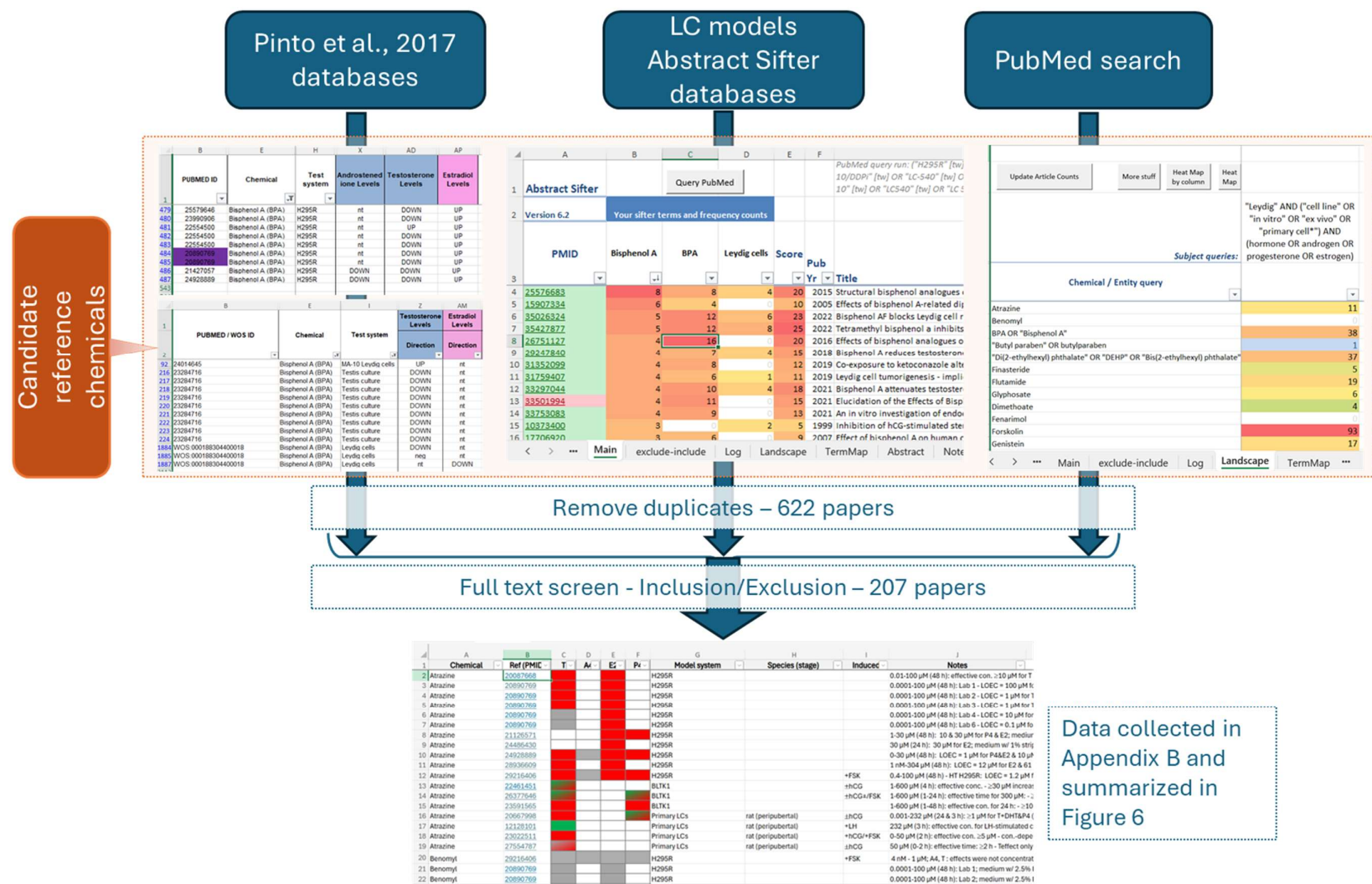
	STIMULATION					ENZYMES							CHOLESTEROL TRANSPORT			
	hCG	LH	cAMP analog	Hydroxy CHOL	Forskolin	CYP11A1	CYP17A1	CYP19A1	AKR1C3/6/14	HSD17B	HSD3B	SRD5A1	STAR	TSPO	CHOL transport	Lipid droplets
Primary	38	39	20	11	26	25	16	2	3	10	17	7	31	3	2	1
LC-like	1	2	3	2	6	4	5	0	0	3	6	0	6	0	0	2
H295R	0	0	0	0	47	16	20	17	0	9	18	0	16	0	0	0
MA-10	12	7	13	4	10	14	5	1	1	3	10	2	13	2	1	2
TM3	4	0	0	0	0	5	5	0	1	3	7	0	6	0	0	1
mLTC1	8	1	1	0	1	1	2	1	0	0	0	0	3	0	0	0
BLTK1	3	1	1	0	1	2	2	2	0	2	2	2	2	0	0	0

	HORMONE													
	P5	P4	DHEA/DHEA-S	A4	A5	T	E1/E2/E3	DHT	17-OHP5	17-OHP4	ADIOL	AN	DHP	5α-A
Primary	8	9	1	2	0	91	6	5	1	0	4	1	0	3
LC-like	1	4	0	1	0	6	0	1	0	0	0	1	0	0
H295R	20	40	24	29	2	67	61	6	14	18	1	2	1	1
MA-10	1	26	0	1	0	8	3	1	0	0	0	0	0	0
TM3	0	0	0	0	0	17	0	0	0	0	0	0	0	0
mLTC1	0	5	0	2	0	6	0	0	0	2	0	0	0	0
BLTK1	0	3	0	0	0	3	1	0	0	0	0	0	0	0

SUPPLEMENTARY FIGURES

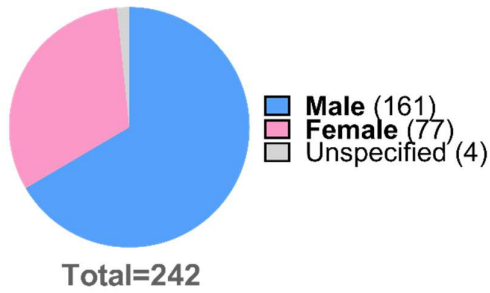


Supplementary Figure S1: Distribution of publication years for all studies included in the review, based on general keyword-based searches of *in vitro* models for testicular steroidogenesis.

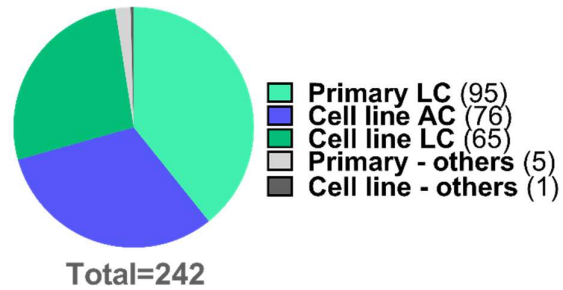


Supplementary Figure S2: Flowchart illustrating the study selection process for targeted searches (Search Strategy 2), focusing on chemical-specific effects on hormone levels in *in vitro* models of male steroidogenesis.

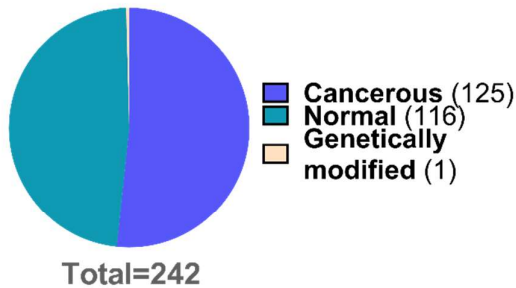
A. Sex



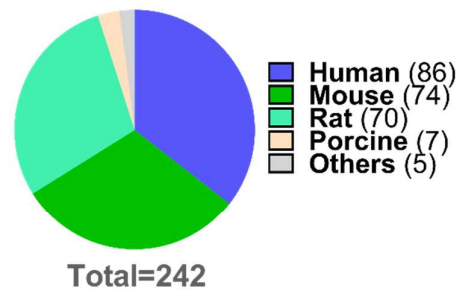
B. Cell type



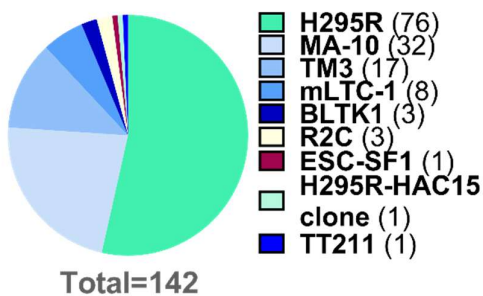
C. Cell normality



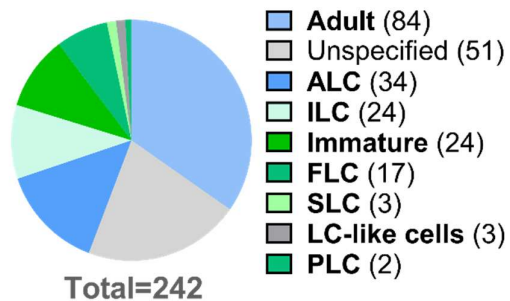
D. Species



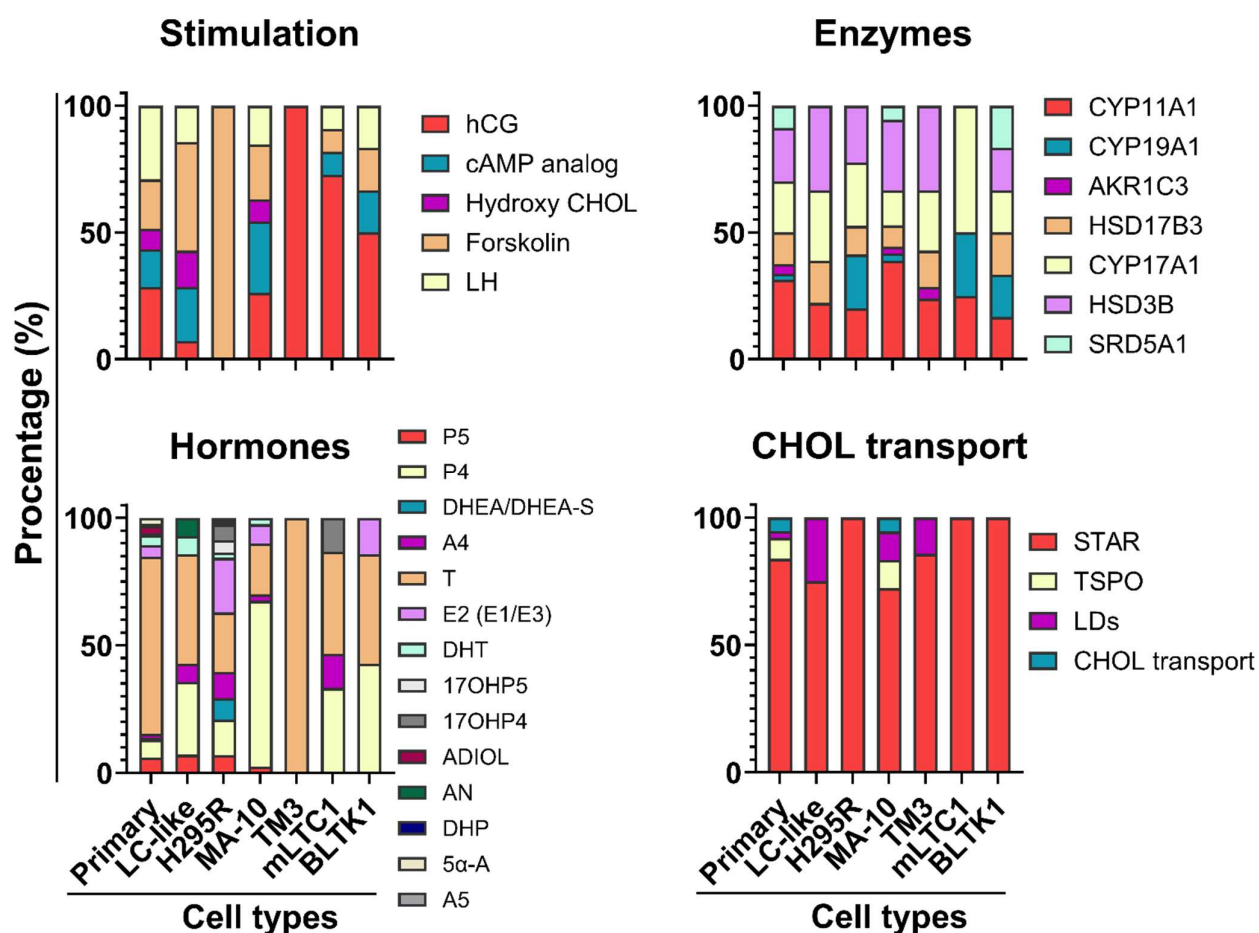
E. Cell line



F. Dev. phase

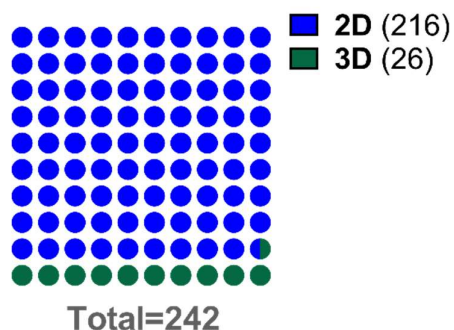


Supplementary Figure S3: Characteristics of *in vitro* models used to investigate the effects of selected chemicals on hormone levels during male steroidogenesis, identified through targeted searches (Search strategy 2). AC, adrenal cell; F, female; LC, Leydig cells; M, male.

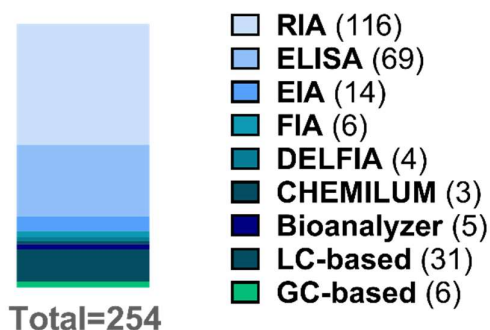


Supplementary Figure S4: Characteristics of *in vitro* models investigating the effects of selected chemicals on hormone levels during male steroidogenesis, identified through targeted searches (Search strategy 2). The figure summarized agents used to stimulate steroidogenesis, steroidogenic enzymes and hormones evaluated, and parameters assessed for cholesterol transport. The number of studies addressing each parameter is provided in **Supplementary Table S3**.

A. Culturing



B. Hormone analysis



Supplementary Figure S5: Culture conditions of *in vitro* models (A.) investigating the effects of selected chemicals on hormone levels during male steroidogenesis, identified through targeted searches (Search strategy 2), and methods used for hormone analysis (B). 2D, two-dimensional; 3D, three-dimensional; CHEMILUM, chemiluminescence immunoassay; DELFIA, dissociation-enhanced lanthanide fluorescent immunoassay; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FIA, fluorescence immunoassay; GC, gas chromatography; LC, liquid chromatography; RIA, radioimmunoassay.