transatlantic think tank for toxicology

t4 Report*

In Vitro Testicular Toxicity Models: Opportunities for Advancement via Biomedical Engineering Techniques

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Summary

To address the pressing need for better in vitro testicular toxicity models, a workshop sponsored by the International Life Sciences Institute (ILSI), the Health and Environmental Science Institute (HESI), and the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), was held at the Mt. Washington Conference Center in Baltimore, MD, USA on October 26-27, 2011. At this workshop, experts in testis physiology, toxicology, and tissue engineering discussed approaches for creating improved in vitro environments that would be more conducive to maintaining spermatogenesis and steroidogenesis and could provide more predictive models for testicular toxicity testing. This workshop report is intended to provide

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Abbreviations: FDA, Food and Drug Administration; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; HPG, hypothalamic-pituitary-gonadal; LH, luteinizing hormone; OECD, Organization for Economic Cooperation and Development; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals

scientists with a broad overview of relevant testicular toxicity literature and to suggest opportunities where bioengineering principles and techniques could be used to build improved in vitro testicular models for safety evaluation. Tissue engineering techniques could, conceivably, be immediately implemented to improve existing models. However, it is likely that in vitro testis models that use single or multiple cell types will be needed to address such endpoints as accurate prediction of chemically induced testicular toxicity in humans, elucidation of mechanisms of toxicity, and identification of possible biomarkers of testicular toxicity.

Keywords: testicular toxicity, bioengineering, biomedical engineering, in vitro screening

1 Introduction

1.1 Overview of the workshop

This workshop on *in vitro* testicular toxicity models was conceived by Bob Chapin (Pfizer) and organized by James Kim (ILSI/HESI), Jennifer Sasaki (AstraZeneca), and Louise Saldutti (Merck). An opening statement was delivered by Thomas Hartung (CAAT), followed by Aldert Piersma (RIVM), who provided an overview of European activities regarding *in vitro* male reproductive toxicity efforts. Terry Brown (Johns Hopkins School of Public Health) and Paul Cooke (University of Florida) presented overviews of male reproductive physiology, spermatogenesis, and steroidogenesis. Paul Foster (NTP) discussed several paradigm testicular toxicants and noted the variety of mechanisms by which testicular toxicity is induced and where *in vitro* approaches have added value to our understanding. Mary Hixon (Brown University), Elaine Faustman (University of Washington), and Sarah Campion (Pfizer) provided perspectives on past and present *in vitro* testicular toxicity models. Tessa DesRochers (Tufts University), Elizabeth Loboa (University of North Carolina at Chapel Hill/North Carolina State University), and Hanry Yu (National University of Singapore) provided biomedical engineering perspectives, discussing advances in the areas of kidney, bone, and liver tissue engineering, respectively. David O'Dowd (Draper Lab, Cambridge, MA) presented examples of applications of biomedical engineering approaches in industry. Following the technical presentations, William Kelce (Novan Therapeutics, Durham, NC) led interactive discussions between the speakers and the audience that elicited lively discussion among academic, government, and industrial participants. In closing, Elizabeth Maull (NIH/NIEHS), Suzanne Fitzpatrick (FDA), and Meta Bonner (EPA) discussed available funding opportunities that could support future research in this area.

1.2 Historical aspects of *in vivo* safety evaluation

Global agreement on hazard and risk assessment of chemicals and pharmaceuticals has been pursued by harmonizing Organisation for Economic Cooperation and Development (OECD) and International Conference on Harmonisation (ICH) protocols and testing approaches. Test guidelines address the range of endpoints such as systemic, developmental, and reproductive toxicity and carcinogenicity, with heavy reliance on animal data. While this approach is effective for evaluation of chemical and pharmaceutical safety, it is costly, time-consuming, and requires large numbers of experimental animals. In response, methods varying in complexity from *in silico* prediction tools and biochemical assays to cell/organ culture methods can offer cost-effective and time-saving alternative approaches to *in vivo* testing. However, their implementation in hazard and risk assessment paradigms, replacing or reducing animal studies, has lagged behind due to uncertainties as to the predictive capacity of these reductionist methods. Furthermore, their "applicability domain" – in view of the biological space covered and the chemical classes for which they are predictive – has been a subject of continuous debate.

Since the landmark publication by Russell and Burch (1959), the 3R paradigm of Replacement, Reduction, and Refinement of animal experimentation has been a leading theme in the development of alternative methods. For complex biological systems such as the reproductive system, it has been recognized that 1:1 replacement of an *in vivo* animal study by animal-free methods is unlikely. However, combinations of alternative tests in a tiered and/or battery approach may provide improved toxicity prediction. This approach was tested in the European ReProTect collaboration (Hareng et al., 2005) in which ten carefully-selected compounds were tested in ten assays covering different aspects of the reproductive cycle (Schenk et al., 2010). This pilot study showed that a combination of complementary assays could improve prediction as compared to single assays. In the US, the ongoing ToxCast project at the US Environmental Protection Agency (EPA) (Dix et al., 2011; Knudsen et al., 2011) represents a large-scale investment towards integrated *in vitro* toxicity assessment, in which hundreds of high throughput assays have been aligned to characterize toxicity profiles of chemicals. Complementary to this, the Human Toxome Project[1] has started mapping pathways of toxicity (Hartung and McBride, 2011) using endocrine disruption as a test case.

Reduction of animal use may be achieved by optimizing animal studies and their alignment in a testing strategy, together with alternative methods as prescreening tools. For instance, the recently approved extended one-generation reproductive toxicity study protocol (OECD TG 443), when replacing the OECD TG 416 two-generation reproductive toxicity study, could reduce

[1] http://humantoxome.com

animal use by 40% compared with OECD TG 416, and overall animal use in the entire Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation by 15% (Bremer et al., 2007; Hartung and Rovida, 2009; Piersma et al., 2011). Attempts also are underway to design innovative testing strategies making optimal use of alternatives (van der Burg et al., 2011; Hartung et al., 2013).

Refinement of hazard assessment by elucidating mechanisms of action is another approach where alternative assays can provide important additional information to inform interspecies extrapolation and assess the human relevance. The European and ToxCast collaborations mentioned above provide good examples of relevant approaches in this area. In addition, the US National Academy of Sciences (NAS) vision on Toxicology in the 21st Century has generated the concept of a restricted number of biological pathways that could be perturbed to result in adverse effects (NRC, 2007). The NAS in 2000 (NRC, 2000) identified the extraordinary genomic conservation of signaling pathways for development and reproductive response pathways and served as the basis for identification of potential pathways for toxicity assays in *in vitro* and non-vertebrate assays. The identification of these pathways is the current challenge. Their representation in alternative assays could provide the basis for an *in vitro* testing strategy. However, it should be realized that the entire organism is more than the sum of its parts and, therefore, animal studies will remain necessary for the immediate future.

Awareness of the need to implement alternative methods was increased by novel chemical safety legislation in Europe and the implementation of REACH (European Commission, 2007). The European Chemicals Bureau has published an estimation of predicted experimental animal use arguing that 65% of test animal usage for REACH would be for reproductive and developmental toxicity assessment alone (van der Jagt et al., 2004) based on counting the pups used in the multigeneration studies. This large number of animals turns reproductive and developmental toxicology into a high priority area for developing alternative methods.

Several analyses of existing reproductive toxicity study databases have shown that the testis often is among the most sensitive organs to exogenous exposure, driving the determination of lowest observed adverse effect level (LOAEL), which is the starting point for risk assessment (Bremer et al., 2007; Dang et al., 2009; Martin et al., 2009). Therefore, the testis certainly is a priority organ for the development of alternative assays. Although the ultimate goal would be to design *in vitro* cultures that could successfully recreate the key aspects of spermatogenesis and steroidogenesis, few of the current mammalian testis cell culture methods support or yield mature germ cells (Sato et al., 2011).

1.3 Application of *in vitro* testicular toxicity models to environmental chemical and pharmaceutical safety testing

To date, no systems produce germ cells in sufficient quantity or in consistently repeatable units across assay replicates (e.g., within a 48-well tissue culture plate) to be useful in routine toxicity screening or mechanism of action assessment. However, were such platforms available, they would be immediately applicable in pharmaceutical discovery and development, as in the case where a lead compound produces testis damage. The screening assay could be used to assess the toxicity of possible backup compounds. Additional applications include elucidation of the mechanisms of toxicity and/or identifying biomarkers for nonclinical and clinical use. Such assays also could be of great value for initial first-tier screening of environmental compounds, where the number of chemicals with unknown biological activity is vastly larger than the resources to test them in animals. For example, based on the Toxic Substances Control Act (TSCA, 1976) there are more than 75,000 chemicals in use in the United States that ultimately make it into the environment. Most of these chemicals have limited or no toxicological evaluation. For such a large number of compounds, toxicity characterization via current approaches that rely heavily on animal testing would cost millions of dollars and take several years per compound. Several initiatives are tackling these issues, including the ILSI/HESI tier-based approach for toxicity screening and testing prioritization of agricultural chemicals (Barton et al., 2006; Carmichael et al., 2006), while others aim to characterize toxicity solely via *in vitro* models, such as the NRC initiative for developing *in vitro* approaches to chemical toxicity characterization (NRC, 2007). In the EU in 1981 there were an estimated 100,000 chemicals on the market. For most of these chemicals there are limited or no risk assessment data, and the lack of data on high volume chemicals (>1,000 tons per year) is of particular concern[2]. In 2007 in the European Union (EU), REACH chemical legislation was established to consolidate regulations on new and existing chemicals to define a more consistent approach to chemical regulation. Because testing was now required for new (post-1981) chemicals with production volumes ≥10 kg, this new legislation encouraged use of existing chemicals. Subsequently, research and development of new chemicals has been greatly inhibited, with only ~3000 new chemicals introduced to the market since REACH legislation was enacted. Even with the reduction in newly introduced entities, toxicity characterization is still needed in the EU and globally for thousands of marketed chemicals, highlighting the need for well-designed *in vitro* assays that can screen and prioritize chemicals of potentially high safety risk.

Similarly, the pharmaceutical industry has struggled to identify and implement effective early safety screening approaches to offset the rising cost of pharmaceutical development (Scannell et al., 2012; McKim, 2010). While testicular toxicity may not be the leading contributor to drug candidate attrition, identification of testicular lesions in repeat dose Good Laboratory Practice (GLP) animal toxicology studies of four weeks or longer in duration, but not during initial one- or two-week non-GLP dose-range finding studies, can incur significant costs, particularly if clinical trials already have been initiated (Sasaki et al., 2011). Furthermore, in contrast to safety liabilities such as hepatotoxicity, in

[2] http://www.hse.gov.uk/reach/resources/factsheet.pdf

which toxic insult in both animals and humans can be readily monitored via well-established endpoints such as increases in blood levels of liver-specific transaminases, attempts to identify a sensitive and specific translational marker for testicular injury have been largely unsuccessful (Elkin et al., 2010).

With appropriate caveats, it sometimes is possible to predict the adverse outcomes of a structurally related chemical entity based on the *in vitro* signature that has been constructed for the structural class, and that is biologically related to *in vivo* outcomes such as specific morphological changes, gene expression signatures, or hormonal responses. The availability of *in vitro* predictive screening assays for testicular toxicity would allow drug discovery units to screen quickly for testicular liability among potential drug candidates using small amounts (mg) of compound. As the testicular toxicity of a candidate may be further modulated by distribution, metabolism, or excretion characteristics, *in silico* or *in vitro* partitioning or transport models may be useful, in combination with *in vitro* testicular toxicity data, to assess the dose-response effect of the compound within the rodent and non-rodent species or human male reproductive tract.

2 Testicular toxicity outcomes in human versus nonclinical regulatory toxicology species

Before considering available *in vitro* testicular toxicity® models, the ability of animal models to predict the human toxicity potential of pharmaceuticals and chemical products should be considered. While *in vivo* safety testing in nonclinical models is expected to identify and predict male reproductive toxicity potential in humans, the examples below illustrate that a testicular toxicity signal during preclinical development in one or more species needs to be carefully assessed prior to determining the relevance for human risk assessment.

With the possible exception of cytotoxic oncolytics and reproductive hormone agonists/antagonists, the value of animal models in predicting testicular toxicity in man following exposure to pharmaceuticals is difficult to establish given the uncertainty around data interpretation and the relatively sparse data available in both animals and humans. In terms of data interpretation challenges, for example, it is plausible that testicular toxicity observed in a single animal species can be considered potentially relevant to humans, unless mitigating circumstances are demonstrated (Creasy, 2002). It has also been recognized that toxicity findings in a single animal species may have low predictive value (Olson et al., 2000). Olson and colleagues demonstrated that over a wide range of organ/system toxicities, the value of animal models for predicting human toxicity increased when toxicity is observed in multiple species and that single species findings may not always translate into human toxicity. However, in terms of toxicity to the reproductive system, it should be noted that reproduction is a highly conserved process in mammals, which may increase confidence that animal findings will translate to humans as compared to other organ systems. Olson et al. (2000) identified 150 compounds under pharmaceutical development that were known to produce human toxicity in one or more organ system, and then examined the animal toxicology reports for these 150 compounds to determine the predictive value of the animal data. The true positive concordance rate was 70% for one or more preclinical animal model species (including rodent and nonrodent), showing the target organ toxicity that also was seen in the same human organ system. For the remaining 30% of human toxicities, there was no relationship between toxicities seen in animals and those observed in humans. Concordance with the human toxicities dropped markedly when only a single species demonstrated similar toxicity. Concordance fell to 27% when only one nonrodent species demonstrated similar toxicity and to 7% when only one rodent species demonstrated similar toxicity.

In 2011, the US Food & Drug Administration (FDA) approved 24 new molecular entities (NME) and 6 biologic license applications (BLA). Following review of the product labels and/or drug approval packages, there were 8 of these approved drugs that exhibited evidence of testicular toxicity in at least 1 species, with 2 drugs exhibiting findings in 2 species (Tab. 1). Three of these 8 drugs (Daliresp®(roflumilast), Victrelis™ (boceprevir) and Incivek™ (telaprevir)) had dedicated clinical reproductive studies that failed to demonstrate detectable human reproductive toxicity. See Table 1 for an exposure comparison between human and nonclinical species.

During its nonclinical development, Daliresp® produced an increased incidence of tubular atrophy, degeneration of the testis, and sperm granulomas in the epididymides in rats. Daliresp® also exhibited effects on the male reproductive tract in dogs and mice, but not in hamsters and monkeys. Interestingly, there were no effects of Daliresp® on semen parameters or reproductive hormones in a 3 month clinical study[3].

In nonclinical studies with Victrelis™ , drug-exposed rats exhibited decreased fertility due to testicular degeneration. Testicular findings in the rat were not associated with alterations of follicle stimulating hormone (FSH), luteinizing hormone (LH) or testosterone, and generally consisted of lower epididymal, prostate, and testes weights. In the epididymides, findings included luminal cell debris and/or hypospermia, while in the testes, Sertoli cell vacuolation, depletion and/or degeneration of spermatocytes and spermatids, and atrophy of the seminiferous tubules were observed; signs of reversibility of these testicular toxicity findings were observed following a 2 month treatment-free period. Testicular toxicity was not observed in immature or sexually mature cynomolgus monkeys, dogs, or mice exposed to Victrelis™ . In addition, clinical monitoring of male subjects for surrogate markers of testicular function, which included inhibin B and semen analysis, indicated that Victrelis™ exhibited no evidence of testicular toxicity in men[4,5].

[3] http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/022522s000lbl.pdf

[4] http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/202258Orig1s000TOC.cfm (accessed in 2012).

[5] http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Label_ApprovalHistory#apphist (accessed in 2012)

Tab. 1: Testicular toxicities observed in 2011 US FDA approved new molecular entities and biologic license applications

Proprietary name	Indication	Non-clinical data	Clinical data
Adcetris	Lymphoma	Seminiferous tubule degeneration, Sertoli cell vacuolation, reduced spermatogenesis and aspermia observed in rats at 3 times the maximum recommended human dose (MRHD).	No data
Daliresp	Chronic Obstructive Pulmonary Disease	Seminiferous tubular atrophy, testis degeneration, spermiogenic granulomas in the epididymides and dysspermia observed in rats at 29 times the MRHD; reproductive tract changes in dogs and mice, but not in hamsters and monkeys; no effects on semen parameters or reproductive hormones in a 3 month clinical study (Forest Pharmaceuticals Inc., 2011; FDA Summary Review NDA 022522).	No effects on semen parameters or reproductive hormones during 3 month treatment and 3 month treatment-free periods.
Eylea	Macular Degeneration	Changes in sperm morphology and motility observed in monkeys at 1500 times the MRHD.	No data
Firazyr	Acute Angio Edema	Testicular atrophy, reduced prostate gland secretion, and decreased testosterone observed in rats administered 5 times the MRHD. Reduced sperm count, atrophy of the testes and prostate, and decreased testosterone observed in dogs at 30 times the MRHD.	No data
Incivek	Hepatitis C Virus	Decreased % motile sperm and increased non-motile sperm count observed in rats at 0.3 times the MRHD. Testicular toxicity was not observed in dogs.	Monitoring of LH, FSH, and Inhibin B revealed no evidence of testicular toxicity
Victrelis	Hepatitis C Virus	Decreased fertility in male rats was attributed to testicular degeneration at 1.3 times the MRHD. Testicular degeneration was not observed in mice, dogs, or monkeys.	Monitoring of Inhibin B and semen revealed no evidence of testicular toxicity.
Xalkori	Metastatic Lung Cancer	Testicular pachytene degeneration observed in rats at 3 times the MRHD.	No data
Zytiga	Prostate Cancer	Testicular atrophy, aspermia/hypospermia and hyperplasia of the reproductive tract observed in rats and monkeys at 1.1 and 0.6 times the MRHD, respectively. This was consistent with the antiandrogenic pharmacological activity of the drug.	No data

Similarly, in nonclinical studies for rats administered Incivek™ , there was an adverse effect on sperm parameters and evidence of degenerative testicular toxicity6. In rats, microscopic testicular lesions included germinal epithelial degeneration, germ cell degeneration/necrosis, tubule degeneration, enlarged residual bodies, retained spermatids and vacuolation of Sertoli cells. Rats exposed to Incivek™ also exhibited a decrease in percentage motile sperm as well as an increase in the number of nonmotile sperm. In general, effects on the male rat reproductive system appeared to be reversible. These testicular findings were not observed in dogs exposed to Incivek™ , suggesting that the testicular toxicity in rat may be species-specific. Furthermore, there were no adverse effects on reproductive hormones (inhibin B, LH, and FSH) in human subjects following exposure to Incivek™ .

As described in Sasaki et al. (2011), Cialis®(tadalafil) had no adverse effects on fertility in rats or on the male reproductive system in rats or mice at doses that produced an exposure of 11-14 times the highest recommended clinical dose (20 mg/day; Hellstrom et al., 2003; Lilly, 2010). However, dogs given tadalafil daily for 6 and 12 months exhibited treatment-related non-reversible degeneration and atrophy of the seminiferous tubular epithelium in the testes (FDA, 2003, Application Number 21-368). Based on the findings in the dog, three randomized, double-blinded, placebo controlled clinical trials were conducted with tadalafil to assess the risk to male reproductive function. In the first two trials (Hellstrom et al., 2003), healthy men or men with mild erectile dysfunction (ED) were enrolled to placebo, 10 mg tadalafil (Study 1), or 20 mg tadalafil (Study 2) once a day for 26 weeks. In Study

6 http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/201917Orig1s000TOC.cfm (accessed in 2012)

Tab. 2: Testicular toxicities observed in non-pharmaceutical substances

Substance	Animal data	Human data
DBCP	Rats exhibited decreased testes, prostate gland, and seminal vesicle weights; elevated LH and FSH (Warren et al., 1984)	Azoospermia or oligospermia and raised serum levels of FSH and LH (Whorton et al., 1977)
TCDD	Exposure of adult rats had no reproductive findings; however, postnatal exposure resulted in altered testes and accessory gland weight, testicular morphology, spermatogenesis, and subsequently fertility (reviewed by Eskenazi and Kimmel, 1995)	Decreased sperm count, altered reproductive hormones (reviewed by Eskenazi and Kimmel, 1995; Mann, 1997)
DEHP	Rats exhibited reduced testes, epididymal and prostate gland weights; reduced epididymal sperm density and motility; reduced testosterone and increased LH and FSH (Agarwal et al., 1986).	Reduced sperm motility and sperm DNA damage (Hauser et al., 2005, 2007)
2,4 Toluenediamine	Rats exhibited decreased number of sperm in the seminiferous tubules and cauda epididymides (Thyssen et al., 1985).	Decreased sperm count, altered sperm morphology and aspermatogenesis (Tchounwou et al., 2003)
Carbon Disulfide	Rats exhibited decreased epididymal sperm counts and testosterone levels (Tepe and Zenick, 1984).	Decreased spermatogenesis, FSH, LH and libido (Schrag and Dixon, 1985).
EGME	Mice exhibited decreased testes weight, tubular atrophy, and germinal epithelium degeneration (Foster et al., 1983; Creasy and Foster, 1984; reviewed by Hardin, 1983).	Decreased testis volume, sperm motility, concentration, altered morphology; increased FSH (Veulemans et al., 1993)
Lead	Rats exhibited impaired spermatogenesis, decreased sperm motility and reduced concentrations of androgens (Apostoli et al., 1998).	Decreased sperm count and motility, hypospermia, decreased testosterone (Apostoli et al., 1998)
Chromium	Rats exhibited testicular atrophy and a reduction in epididymal sperm number (Ernst, 1990).	Decreased sperm concentration and motility; altered sperm morphology (Danadevi et al., 2003)

DBCP = 1,2-dibromo-3-chloropropane; TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin; DEHP = di-2-ethylhexyl phthalate; EGME = ethyl glycol monoethyl ether

3 (Hellstrom et al., 2008), healthy men or men with mild ED were randomized to receive placebo or tadalafil 20 mg daily for 9 months. Measurements to assess male reproductive effects included semen sperm concentration, total sperm number per ejaculate, percent abnormal sperm morphology, percent motile sperm, and serum testosterone, LH and FSH. No adverse effects were observed on sperm or hormone parameters. These three clinical studies demonstrated no adverse effects of tadalafil on spermatogenesis or hormones related to testicular function in men following up to 9 months of daily exposure. The findings in men were consistent with the nonclinical results in rats and mice. The findings in the dog may suggest that the dog was a poor model to assess the risk for testicular injury following treatment with tadalafil.

In contrast to pharmaceuticals, the non-drug chemical database may be more limited for assessing the correlation between human and animal testicular toxicity. Ulbrich and Palmer (1995) published the results of a literature survey assessing various methods for detection of effects on male reproduction. In comparing the animal and human data, Ulbrich and Palmer identified 42 compounds with reported data on human male reproductive effects; however, only 8 of these compounds fell within the non-drug (pesticide, industrial chemical, solvent, and metal) categories (Tab. 2). Of these 8 non-drug molecules with both human and animal data, 6 (dibromochloropropane (DBCP), di-2-ethylhexyl phthalate (DEHP), 2,4-toluenediamine, carbon disulfide, ethylene glycol monomethyl ether (EGME) and lead) had common reported effects on spermatogenesis or sperm counts in humans and rats or mice. In the remaining two cases (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and chromium), findings in adults for effects on spermatogenesis or spermatogenic-related endpoints were observed in both human and animal models, or the available data were limited/equivocal (Tab. 2). The summation indicates that for these few (and generally quite toxic) non-drug chemicals, the reported effects on spermatogenesis and sperm production in men were generally correlated with reported similar findings in rats and/or mice.

3 Background information on the testis

3.1 Testis physiology

To enable an appreciation of the complexity of the testis, some aspects of basic physiology are reviewed here. Readers also may want to refer to the following books for additional review: Physiology of Reproduction (De Krester and Kerr, 1988), Leydig Cell in Health and Disease (Payne and Hardy, 2007), Sertoli Cell Biology (Boekelheide et al., 2005), Methods in Reproductive Toxicology (Parvinen et al., 1993). Because many of the existing models of spermatogenesis use cells from immature or even neonatal animals, any model that involves *in vitro* maturation of these cells will be accommodating this biology by default, and being aware of such changes is prudent.

In the human 46,XY male fetus, the Sry gene directs the undifferentiated gonad to become a testis. Beginning around 4 weeks of gestation in the human male fetus, the germ cells migrate to the gonadal ridge and the primitive sex cords develop into the seminiferous tubules that envelop the primitive germ cells and Sertoli cells. The secretion of Müllerian inhibiting substance (MIS)/anti-Müllerian hormone (AMH) by the Sertoli cells causes the Müllerian ducts to regress, while Leydig cells in the interstitium of the developing testis secrete testosterone to stimulate the development of the Wolffian ducts that give rise to the epididymides, vas deferens, seminal vesicles, and ejaculatory ducts (Fig. 1). Testosterone, via conversion to its more active 5-α reduced metabolite, dihydrotestosterone (DHT), induces the differentiation and development of the prostate and external genitalia, including the phallus and scrotum. The human fetal period of testosterone synthesis from around 8 weeks until about 20 weeks of gestation is considered critical for the embryonic development of the male reproductive tract and external genitalia and, therefore, disruption of androgen biosynthesis and/or action during this period can have deleterious effects on male development.

The timing of testicular descent varies among species, with this event occurring prior to birth in humans, rabbits, and horses but after birth in rats, mice, and dogs. Testicular descent is mediated in part by the actions of the Leydig cell products, testosterone, and insulin-like 3 (Insl3) protein. In the human, testosterone synthesis by the testis occurs again during a short 6-month period immediately following birth when testosterone is considered to be essential for programming of male development (early postnatal period) and then resumes at puberty for maturation of the male reproductive tract and development of secondary sexual characteristics (pubertal period).

Early testicular synthesis of testosterone in the fetus is stimulated by human chorionic gonadotropin (hCG dependence), followed by maturation of the fetal hypothalamic-pituitary-gonadal (testicular) (HPG) axis that integrates the function of the male endocrine system. Hormonal, neural, and environmental cues regulate the synthesis and release of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the hypothalamic-pituitary portal system through which it reaches its receptors on gonadotrophs in the anterior pituitary to stimulate the synthesis and release of LH and FSH from the anterior pituitary into the peripheral circulation. FSH secreted by the pituitary gland binds to its receptors on Sertoli cells and promotes spermatogenesis via Sertoli-germ cell communication; LH also secreted by the pituitary gland binds to its receptors on Leydig cells and stimulates the biosynthesis of testosterone. The secretion of testosterone by Leydig cells provides negative feedback signals at the levels of the hypothalamus and pituitary to down-regulate the release of GnRH and LH/FSH, respectively. The yin-yang nature of the feedback system predicts the observed pulsatile secretion of GnRH, LH-FSH, and testosterone. In addition, inhibin B produced by Sertoli cells in the testis provides further feedback inhibition of FSH release by the pituitary. Within the testis where the local concentration of testosterone is very high, a significant portion of this androgen is bound to androgen binding protein (ABP) secreted by Sertoli cells.

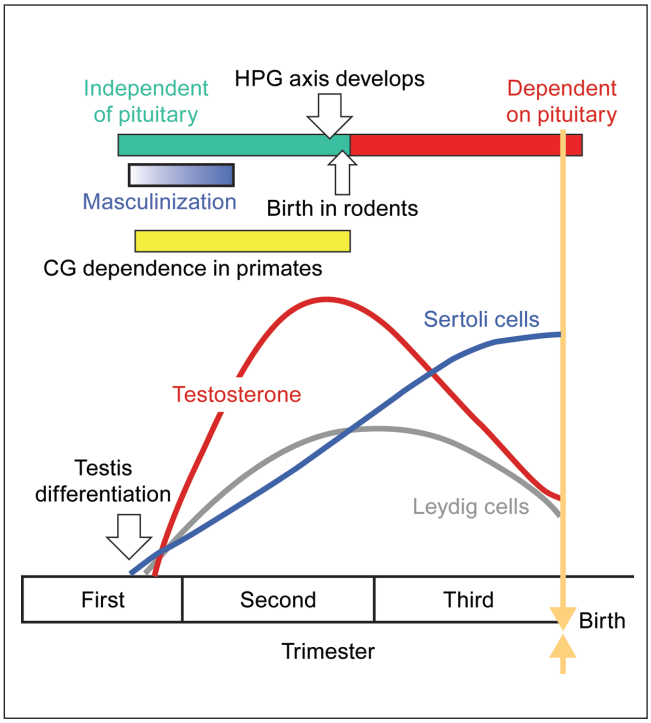

Fig. 1: Schematic representation of the regulation of testicular function during fetal development in the mammal
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Androgen receptors (AR) are expressed in Leydig, Sertoli, and peritubular myoid cells, and respond to testosterone to maintain testicular function, including the androgen-dependent stages of spermatogenesis. Sertoli, Leydig, and germ cell numbers are dependent upon FSH and/or androgen activity in early life, and their numbers are decreased in AR or FSH receptor knockout mice.

Spermatogenesis following puberty appears as a highly conserved pattern of germ cell maturation referred to as the cycle of the seminiferous epithelium that begins with spermatogonia localized to the basement membrane between adjacent Sertoli cells and evolves through spermatocytes and spermatids to mature spermatozoa that are released from their contact with Sertoli cells into the lumen of seminiferous tubules (Fig. 2). The blood-testis barrier formed by tight junctions between adjacent Sertoli cells prevents large molecules from passing directly from the blood into the lumen of the seminiferous tubule and separates spermatogonia in the basal compartment from spermatocytes and spermatids in the adluminal compartment.

Since steroidogenesis is another key function of the testis that needs to be captured *in vitro*, some consideration of this process is merited. The production of testosterone as a result of steroidogenesis occurs in the Leydig cell. Testosterone mediates numerous functions throughout the life cycle of the male, including: 1) the differentiation and development of the male reproductive tract, 2) the neonatal organization of androgen-

dependent target tissues, assuring their appropriate response to androgens later in puberty and adult life, 3) the masculinization of the male at puberty, 4) the activation of growth and function of androgen-dependent target organs in adults, 5) the permissive effects on potency and libido during adult life, and 6) the senescent androgen-dependent changes accompanying Leydig cell dysfunction with advancing age (Ewing et al., 1983; Hall et al., 1988; deKrester et al., 1988; Dufau, 1988).

The Leydig cell lineage has been studied most extensively in the rat testis. Interestingly, a fetal Leydig cell is not an immature Leydig cell, and an immature Leydig cell is not a young adult Leydig cell. Fetal Leydig cells do not give rise to immature or adult Leydig cells, and appear to persist in the adult testis as a minor Leydig cell population (Kerr and Knell, 1988). The immature rat Leydig cell population arises from mesenchymal-like precursors in the interstitium (de Krester et al., 1988). This occurs primarily during postnatal days 14-28, accounting for approximately 50% of the Leydig cells that eventually will populate the adult testis (Hardy et al., 1989); the remaining 50% are derived from mitosis of these newly differentiated cells during postnatal days 28-56 (Hardy et al., 1989, 1990). These newly formed "immature" Leydig cells are capable of LH-induced androgen synthesis. However, the principal androgens secreted are 5α-dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol; Steinberger and Ficher, 1968; Ficher and Steinberger, 1971; Coffee et al., 1971; Goldman and Klingle, 1974; Rosness et al., 1977; Chubb and Ewing, 1981; Vreeburg et al., 1988; Myers and Abney, 1990). The synthesis and secretion of DHT and 3α-diol by immature Leydig cells is the primary feature that distinguishes these cells from "adult" Leydig cells. By approximately postnatal day 56, the immature population of Leydig cells synthesizes and secretes testosterone as the primary androgen, which is characteristic of the adult Leydig cell population. Not only should investigators be aware of changing cell populations throughout the lifespan of the male but also whether each cell population can be successfully cultured is critical for correct data interpretation: special culture conditions are required to maintain steroidogenic capacity in highly purified adult Leydig cell cultures (Klinefelter and Ewing, 1989).

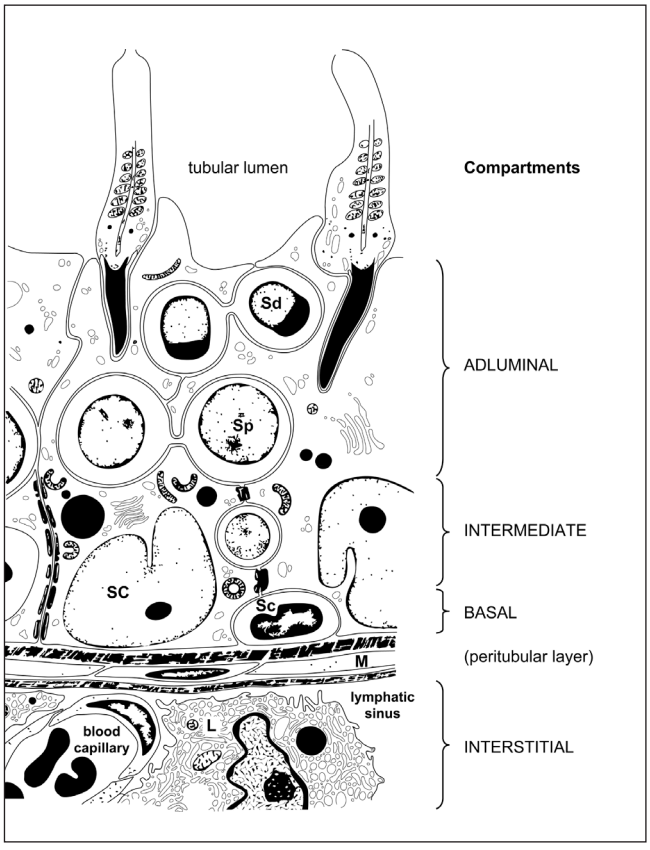

Fig. 2: Compartments of the Testes
From Creasy and Foster, 2002, with permission from Elsevier.

3.2 Targets of testicular toxicity

The overall objective of the reproductive process for the adult male is the production of gametes capable of fertilization and the production of viable offspring. A consideration of the critical events involved in these processes would highlight a large number of potential targets for the action of chemicals upon the male reproductive system (Fig. 3, 4). For a historical perspective on sperm stages see LeBlond and Clermont (1952). While the overwhelming number of chemicals known to affect the male reproductive system appears to do so by directly interfering with the process of spermatogenesis, there are a number of other targets that also can result in adverse male reproductive outcomes. That is to say, fertility is more than just spermatogenesis. Any careful consideration of the use of *in vitro* spermatogenesis for screening for compound toxicity should be aware of the components of male fertility which such a system likely will miss. The most obvious of these would be actions on the hypothalamic-pituitary hormonal control systems. Two examples are dopamine analogues that interrupt the normal secretion of GnRH and estrogen action at the level of the hypothalamus and pituitary that interfere with gonadotropin (LH and FSH) production, adversely impacting normal testicular function. However, there also are chemicals that impair spermatogenesis via indirect mechanisms not specifically related to the HPG axis. For example, nutritional deficits and overexposures to critical vitamins and minerals (e.g., vitamin A and zinc) can perturb normal male reproductive function. Similarly, chemicals with direct effects on the liver (e.g., carbon tetrachloride) can disturb the normal metabolism of sex steroids leading to changes in clearance (predominantly of glucuronide and sulfate conjugates of hydroxytestosterones in the male), indirectly affecting the HPG axis and disrupting male reproduction.

There are more examples of processes important to reproduction that will not be captured in an *in vitro* model of spermatogenesis. For example, most experimental rodents normally employed in reproductive toxicity studies do not show marked seasonality in breeding performance. However, there are dramatic exceptions, such as the hamster, where during the breeding season (or under lab conditions of 14 hours light and 10 hours dark) up to 10% of an adult male's body weight may be testicular tissue. In this species, markedly changing the light:dark cycle to reduce the hours of daylight can influence

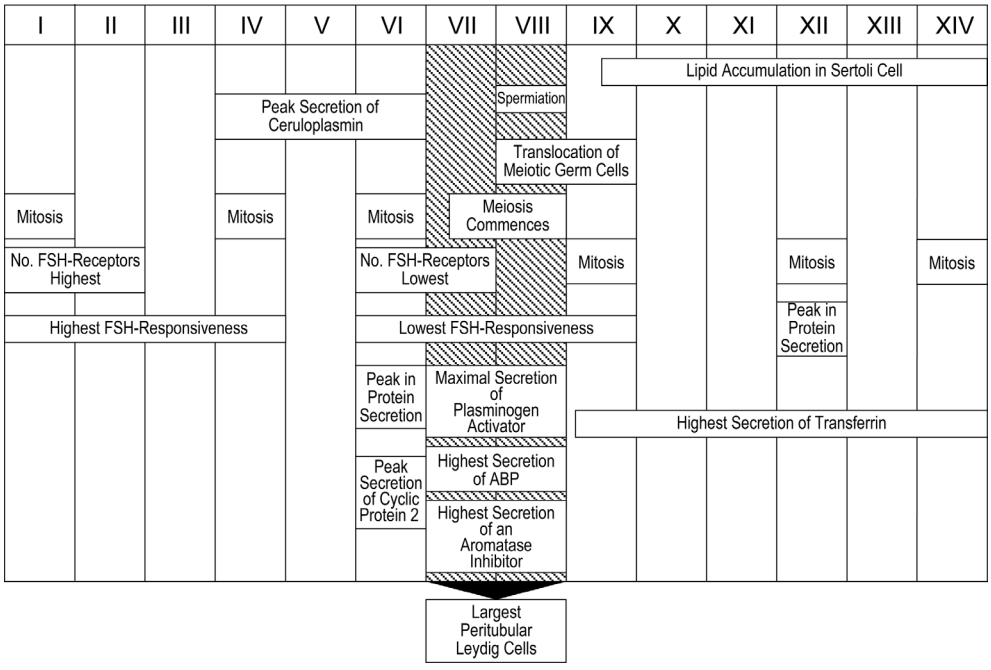

Fig. 3: Stage Specific Changes of the Testes
Adapted from Fritz et al. (1981), LeCroix et al. (1981), Niemi and Kormano (1965), Parvinin (1982), Ritzén et al. (1982), Wright et al. (1981, 1983).

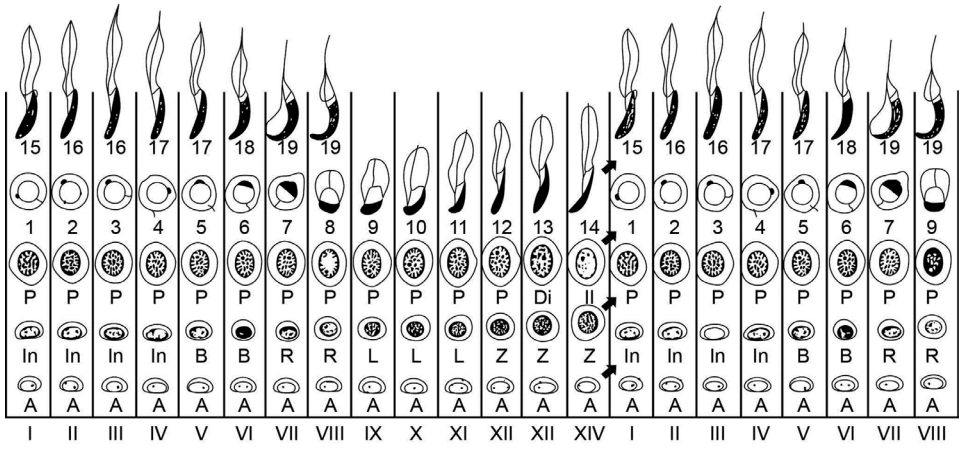

Fig. 4: Diagram illustrating the cellular composition in the 14 stages of the cycle of the seminiferous epithelium in rats where each column represents a stage (denoted by Roman numerals I-XIV)
The stages are defined by the step of development of the accompanying spermatid. Spermatid development is subdivided into 19 steps (denoted by Arabic numbers 1-19) according to the appearance of the acrosome structure, which can be demonstrated using the periodic acid-Schiff-hemotoxylin technique. In a given area of tubule, cellular associations succeed one another in time, proceeding from the left to the right of the diagram. Stage XIV is followed by stage 1 to begin another cycle. One complete sequence of the 14 cellular associations constitutes one cycle of the seminiferous epithelium, whereas the developmental sequence of spermatogonia through spermatocytes to spermatids constitutes the process of spermatogenesis. This also is illustrated in the diagram where, with succeeding cycles (beginning bottom left), type A spermatogonia (A) divide mitotically and differentiate into intermediate type spermatogonia (In) and type B spermatogonia (B). These give rise to spermatocytes, which proceed through the various phases of meiosis: preleptotene or reating (R), leptotene (L), zygotene (Z), parchytene (P), and diakinesis (Di). Meiotic division gives rise to secondary spermatocytes (II) and then to haploid spermatids, which undergo the steps of spermiogenesis (1-19). As the cells proceed through spermatogenesis, they move up through the epithelium and are replaced by another generation of cells, so four generations of cells develop in synchrony. The mature step 19 spermatid is finally released into the tubular lumen during stage VII (from Creasy and Foster, 2002, with permission from Elsevier).

melatonin levels and, through a cascade of signaling responses, cause regression of the testes and concurrent decreases in testicular steroids and mating behavior. This is a normal circumstance in the wild, but care should be taken in laboratory situations to ensure appropriate housing for the conduct of studies on testicular toxicity with this species.

The testis also has a finely tuned circulatory system in mammals, termed the pampiniform venous plexus, designed to shunt the arterial-venous blood supply and aid in scrotal cooling. Some chemicals can actually target this structure and the testicular circulatory system to induce ischemic shock to the testis, again resulting in injury and reduced fertility, with cadmium being an example of an agent that can induce testicular damage via this "indirect" mechanism. While it is unlikely that these features will need to be recapitulated in an *in vitro* model, they do represent indirect mechanisms of toxicity, which we should recognize will be forfeited in an *in vitro* screening system.

Similarly, there are a number of post-testicular targets that can impact male reproductive function. The testis is a major fluid producing organ that makes interstitial fluid between seminiferous tubules and the intratubular fluid. These fluids are essential for the transport of sperm from the seminiferous tubule through the rete testis and into the epididymis. Changes in fluid production and lack of resorption of fluid in the epididymis can create abnormal pressure conditions in both the seminiferous tubules and epididymis, leading to disrupted spermatogenesis. When sperm are released into the seminiferous tubule lumen and proceed to the epididymis, they also can be the target of toxicant action. Chlorosugars and epichlorhydrin both have been shown to inhibit energy metabolism in sperm that prevents them from functioning normally. The specificity of the response was noted, at least in part, based upon inhibition of the specific isoform of lactate dehydrogenase found in testicular germ cells (LDH-C4) that is essential for energy production and consequent sperm motility. Such a mechanism would be unlikely to be captured by an *in vitro* model of spermatogenesis.

More recent evidence concerning the molecular events that drive fertilization would imply that this also may be a potential target for the action of chemicals to disturb male reproduction. We know, for example, of the requirement for the expression of critical cell surface markers by the sperm that facilitate the normal binding of the sperm to the egg surface membrane which, if blocked or not produced, would inhibit fertilization. Lastly, in an examination of potential target sites, there also is the potential for effects on the male genome or epigenome to induce paternally-mediated developmental toxicity in the embryo/fetus. While this is not a common occurrence, it has been documented with a few specific agents (e.g., cyclophosphamide).

There are innumerable avenues by which physiological parameters of the testis can be disrupted, and only some are listed above. There is much more to learn in order to understand the full relevance of the disturbance of particular parameters and the resulting adverse effects on testicular function.

In summary, when designing an *in vitro* model of spermatogenesis, it is critical to remember that such a model will not evaluate all aspects of male fertility. It will intentionally omit some important non-spermatogenesis components of reproduction that have been found to be targets for toxicants. With this caveat in mind, let us now turn to a closer examination of *in vitro* models of spermatogenesis.

4 Past and present state of *in vitro* testing of testicular toxicity

The establishment of an *in vitro* culture system in which spermatogonial stem cells can be maintained and proliferate, undergo meiosis, and complete spermiogenesis reliably and in quantity does not yet exist (Sofikitis et al., 2005). This is because of the difficulty in the isolation and purification of defined subpopulations of germ cells and the establishment of male germ cell lines, our incomplete knowledge of the optimal biochemical and biophysical conditions that promote the survival and differentiation of germ cells during long-term culture, and our general ignorance of all the multi-factorial contributions that regulate spermatogenesis. We know some things, but not enough yet.

The testis has a diverse cell population, and its function is influenced by a variety of physiologic and anatomic contributions. So far, it has been impossible to recapitulate all that complexity *in vitro*. To make the challenges more tractable, investigators have limited the types of testicular cells being studied in the system. A common way to do this involves *in vitro* models comprised of single cell types (e.g., Leydig, interstitial, Sertoli, germ, or peritubular cells). Some investigators prefer *in vitro* models that retain several cell populations so as to recapture the interactions that occur in the intact testis *in vivo*. In fact, it has been observed that expression of testicular toxicity using these simplified *in vitro* models can be different from that found *in vivo* (Ku and Chapin, 1994). We have an expanding appreciation that truly useful *in vitro* models will have to be more complex than in the past. Accordingly, the co-culture of different combinations of somatic and germ cells has become increasingly popular. See Appendix 1 in the supplementary file at http://www.altex-edition.org for references and specifics for single and co-culture cell cultures.

4.1 Representative examples of mixed culture and 3-dimensional models

Pop-off assay

Co-culture of Sertoli and germ cells is a model that has been used for some time. First described by Gray and Beamand (1984), this model involves physical disruption of the testes from 28-day old rats followed by enzymatic digestion and centrifugal sedimentation to isolate small clusters of cells with a high ratio of Sertoli to germ cells. The Sertoli cells attach to the cell culture surface, forming a confluent monolayer to which the germ cells attach. In addition to the Sertoli cells and round germ cells (spermatocytes and spermatogonia), the cultures also contain a few peritubular cells. Testicular cell types that are missing from these cultures include elongated spermatids and the complex interstitial population that includes Leydig cells. In this culture system, testicular toxicants are reported to cause the detachment of germ cells from the Sertoli cell monolayer, and

the number of germ cells released into the media can be quantified. Toxicants also may cause a decrease in the viability of the cells that remain attached; however, the release of germ cells is not simply a result of cell death. Although particular types of toxicants have been predicted very well using this co-culture model, not all testicular toxicants may be detected. Compounds that cause relatively quick and widespread germ cell loss *in vivo*, whether the primary target is Sertoli or germ cells, have been easily detectable in these cultures, while compounds that cause testicular lesions only following long term exposure, or that produce spermatogenic damage primarily through hormonal disruption, or that impair the release of mature spermatids or target dividing cells, are not likely to be detected. Several studies have reported a very good correlation between *in vivo* activity and *in vitro* activity in this co-culture model with different classes of compounds. Increased germ cell detachment was produced by specific phthalates known to cause testicular damage *in vivo*, while no effects were seen with other phthalates that do not affect the testis *in vivo* (Gray and Beamand, 1984). Treatment of Sertoli germ cell co-cultures with 1,3-dinitrobenzene resulted in failure of the Sertoli cell monolayer to maintain the testicular germ cell population, which models the morphological testicular response obtained *in vivo* (Foster et al., 1987). Ethylene glycol monoethyl ether (EGME) had no effect *in vitro*, but its active metabolite methoxyacetic acid (MAA) caused initial degeneration and subsequent loss of the spermatocyte populations from the culture, an effect similar to that seen *in vivo* (Foster et al., 1984). Several other studies have utilized this culture system as an indicator of testicular toxicity (Allenby et al., 1991a,b; Cave and Foster, 1990; Creasy et al., 1988; Gray, 1986).

2-Chamber culture system

Early attempts to culture primary testicular cells on plastic produced less than satisfactory results due to high cell loss resulting in short culture periods, loss of typical cell architecture which was believed necessary for normal cell function (such as formation of Sertoli cell polarity and basal tight junction formation), and the inability to differentiate germ cells *in vitro*. In search of new methods for better results, publications from the labs of Martin Dym at Georgetown (Byers et al., 1986) and Anna Steinberger at the University of Texas Medical School (Janecki and Steinberger, 1986) reported that the use of dual chambered culture wells could overcome some of the limitations previously observed with culturing Sertoli or Sertoli-germ cells on a monolayer plastic surface (Fig. 5). This represented the first step towards a 3D environment and, indeed, it later evolved into supporting three-dimensional complexity *in vitro*.

In essence, this culture system involves the use of a filter-bottom culture insert within a larger culture dish. Originally, this inner well was constructed by attaching a small disc of filter paper to the bottom of a cut off bottom of a polystyrene centrifuge tube (Byers et al., 1986) or a piece from a hollow glass cylinder (Janecki and Steinberger, 1986) mounted on several small "feet" to allow separation from the bottom of the outer well. Scientific equipment manufacturers, such as Millipore and Greiner, quickly sensed a market need and started making products that offered the ability to conveniently perform such cultures consistently from one lab to the next.

There is nothing unique about the isolation of Sertoli and germ cells for culture in the 2-chamber culture system compared to other *in vitro* culture systems, and the general procedures for isolation of Sertoli cells are covered by Hadley et al. (1985). In es-

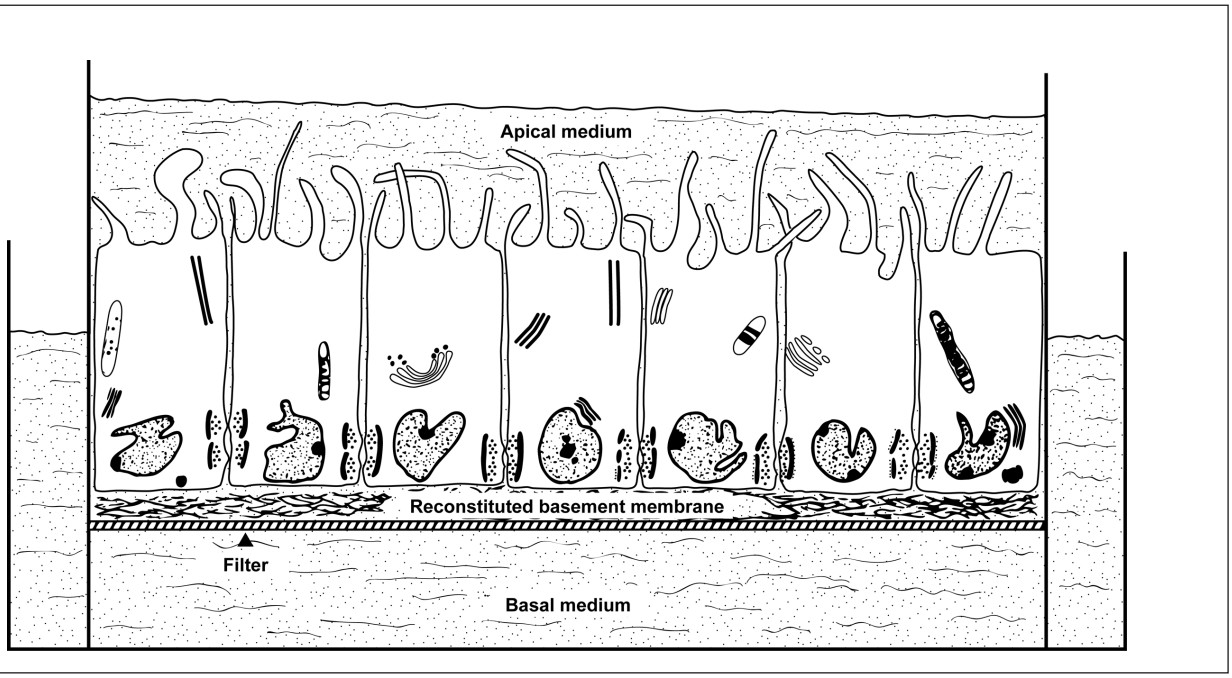

Fig. 5: Schematic diagram of a bicameral culture chamber with Sertoli cells on a Millipore filter coated with Matrigel®
From Djakiew et al. (1986), with permission from the *Journal of Andrology*.

sence, high concentrations of Sertoli cells or mixed Sertoli-germ cell populations acquired from physically disrupted and enzymatically digested testis of usually juvenile rodents were plated on the filter of the inner well. This two-chamber system allowed the addition of, and sampling from, fluid in the inner well, outer well, or both. This was important as investigators began assessing directional secretion from Sertoli cells cultured in the inner well. In addition, when plated at high concentrations, Sertoli cells occasionally formed a confluent layer and developed tight junctions between cells, reminiscent of the blood-testis barrier (BTB). The formation of this BTB *in vitro* within the 2-chamber culture system quickly leads to the incorporation of measuring transepithelial resistance across the inner and outer wells with a Millipore-ERS device (Millipore Co.) as a way to assess the integrity of this "BTB *in vitro*" (Janecki et al., 1990).

Another advance that occurred at this time was the introduction of an extracellular matrix to the culture system in an attempt to more accurately recapitulate the *in vivo* relationship of the seminiferous tubules, where the Sertoli and germ cells are separated from other testicular cells by the lamina propria. In papers by Hadley et al. (1985) and Byers et al., (1986) it was shown that by growing primary Sertoli cells or Sertoli-germ cell co-cultures in or on an extracellular matrix derived from Englebreth-Holm-Swarm tumors in mice, significant improvements could be gained in Sertoli cell survival, germ cell differentiation, and the secretion of the Sertoli cell proteins, androgen binding protein, and transferrin. This product currently is sold as Matrigel®. By incorporating this into the 2-chamber system, it also was possible to measure basal or apical secretion of substances from the Sertoli cells. Hadley et al. (1988) identified the key components that made this 2-chambered system work: the Sertoli cells must be plated at high density with their basal surface in contact with a semipermeable extracellular matrix so that they form basolateral tight junctions, and the cultured cells must be able to engage in cell-to-cell interactions. While not perfect, Hadley et al. (1988) felt that the 2-chamber culture system could reproduce such compartmentalized cultures. Deficiencies in this system, as with other culture systems that attempt to reproduce the *in vivo* testis environment, are summarized at the end of this section.

Much of the published literature utilizing the 2-chamber culture system has focused on exploring the blood-testis barrier and tight junction formation between Sertoli cells and germ cells, junctions that are critically dependent on cell plating density and that can be variable across cultures. These analyses play to the strengths of the system in that the bicameral nature allows biochemical and electrical measurement of the integrity of the cell-cell tight junctions. Compounds can be selectively added to the outer well (which theoretically represents the extra-seminiferous tubule portion of the testis) and migration to the inner well over time can be assessed (Ailenberg et al., 1988; Djakiew et al., 1986). In addition, compounds can be added to the outer well and effects on cell survival and cell-cell tight junctions can be assessed (Chung and Cheng, 2001). Results from these *in vitro* studies have added considerably to the understanding of the molecular and biochemical processes involved with Sertoli-Sertoli interactions and Sertoli-germ cell interactions (see review by Mruk and Cheng, 2004).

While much of the published literature using testicular cells cultured in the 2-chamber culture system has been directed at understanding the physiology of the testis and, more specifically, the seminiferous tubules (Djakiew et al., 1986; Ailenberg et al., 1988; Onoda et al., 1990; Gye, 2003; Kaitu'u-Lino et al., 2007; Wong et al., 2000; Grima and Cheng, 2000; Staub et al., 2000), there are publications dealing with the use of this system to examine the toxicity of various chemicals. For example, Chapin et al. (1990) used the 2-chamber culture system (without intercellular tight junctions) to demonstrate that Leydig cells cultured in the outer well and exposed to tri-o-cresyl phosphate produced an active metabolite that decreased the activity of a target enzyme (nonspecific esterase) inside the Sertoli cells cultured in the inner well, thereby mimicking the effects previously observed *in vivo*. The 2-chamber culture system also has been used by numerous researchers to examine the effects of various chemicals, including cadmium (Janecki et al., 1992; Chung and Cheng, 2001), adjudin (1-(2,4)-dichlorobenzyl-1H-indazole-3-carbohydrazide) (Su et al., 2010), hexane chromium (Geoffroy-Siraudin et al., 2010), and bisphenol A (Li et al., 2009) on the tight junctions. These studies are summarized in Appendix 2 in the supplementary file on www.altex-edition.org. More recently, this system has been used to examine the expression of specific Sertoli and germ cell genes with the hope not only of further understanding the physiology of the Sertoli-germ cell interactions, but also of identifying possible biomarkers for adverse chemical effects (Ryser et al., 2011).

It should be noted that all of the studies mentioned above were performed with known testicular toxicants. No studies were identified where unknowns or an SAR analysis was performed using this 2-chamber culture system for *a priori* assessment of testicular toxicity. Possible reasons for this are included in the summary below covering deficiencies of this system as a way to profile overall testicular effects. Most *in vitro* culture systems are good for addressing specific questions rather than serving as surrogates for the entire organ system. Also, none of the publications used this system with pharmaceuticals; most used industrial chemicals or environmental contaminants.

While there are clear advantages in using the 2-chamber culture system to address specific scientific questions, its significant limitations should be recognized. First, the cells used in these cultures are isolated from juvenile animals rather than adult animals. Also, while germ cell differentiation can be observed, germ cells do not develop into mature sperm or even maturing spermatids. The system is relatively labor intensive, since it uses primary cells; therefore, as currently designed, it will never be used for medium or high through-put screening. Finally, the ability to reliably metabolize compounds to active or inactive metabolites is lacking. These limitations notwithstanding, this system provides a plausible means to address specific questions about compound effects on testicular cell physiology. In fact, the system is commercially available[7] to assess compound effects on spermatogenesis.

[7] www.Kallistem.com

Three-dimensional (3-D) models

Three-dimensional culture approaches are slowly being integrated into many cell culture platforms, including the male reproductive system[8]. Various *in vitro* models have been established from rodent and human tissue to evaluate male reproductive endpoints, and these models include Sertoli/germ cell co-cultures from 10- (Hadley et al., 1985) and 28-day old rats (Gray, 1986), Sertoli/germ cell co-cultures enriched for Sertoli cells from 18-day old rats (Chapin et al., 1988), isolated rat Leydig cells (Yang et al., 2003), and Leydig/Sertoli cell co-cultures from mice (Bilinska, 1989) and humans (Lejeune et al., 1998). These culture systems were used for brief assessments of testicular tissue response, as most were unable to maintain the complex biochemical and physiological systems required in this dynamic tissue (Gregotti et al., 1992; Li et al., 1998).

In several of these co-cultures, pre-coating culture dishes with a Matrigel®/ECM was shown to enhance cell attachment and function (Hadley et al., 1985; Orth et al., 2000). In response to evidence that 3-dimensional extracellular matrix could enhance the relevance of *in vitro* cultures to *in vivo* systems (Cukierman et al., 2001; Hadley et al., 1985), the Faustman lab created a 3-dimensional neonatal rat testicular cell co-culture with a Matrigel® ECM overlay. This co-culture is generated by digestion of testis from five-day old rat pups and contains all major testicular cell types (Yu et al., 2005; Wegner et al., 2013). The presence of Sertoli Cells, Leydig cells, and germ cells has been confirmed with morphological imaging and detection of cell type-specific gene and protein expression (Yu et al., 2009). The ECM overlay radically improved the consistency of the culture, enhancing Sertoli cell attachment and increasing cell communication (Yu et al., 2005). Optimal levels of Matrigel® could be determined because the ECM overlay produced a dose-dependent decrease in stress signaling (as evidenced by decreases in SAPK/JUNK pathway activation) and increases in cell survival signals (ERK and AKT pathways), as well as improved basic cell viability (Yu et al., 2005). These cell cultures in 3-D Matrigel® resulted in stabilized c-Kit expression (marker for functional integrity-spermatogonia-specific proteins). This optimized *in vitro* model of testis development has since been used successfully to distinguish among a series of structurally related, reproductively toxic and non-toxic phthalate esters (Yu et al., 2009), as well as identify a dose-dependent response to cadmium (Yu et al., 2005).

Methods have been developed recently to successfully guide primordial mouse germ cells through meiosis *in vitro*, recapitulating a crucial process of spermatogenesis (Abu Elhija et al., 2012; Nolte et al., 2010; Sato et al., 2011). These promising breakthroughs lay the groundwork for future *in vitro* models of spermatogenesis that could be established from human stem cells. Improvements in consistency and tractability will be needed before they can be widely adopted.

[8] http://3dcellculture.com

4.2 General technical aspects of existing *in vitro* testicular models

Removal of testicular tissue from the whole animal, followed by chemical or mechanical dispersion of the cells for culturing in defined medium produces an "unnatural" environment. Furthermore, testicular cells obtained from tumors or otherwise transformed cells cannot be compared directly to normal testicular cells in the whole animal. For additional discussion of the potential differences between *in vivo* systems versus *in vitro* platforms, readers may refer to the review article by Russell and Steinberger (1989) that highlights differences between *in vivo* Sertoli cells and Sertoli cell cultures. The following known shortcomings must be considered in determining the relevance of data derived from existing *in vitro* testicular models, and they provide excellent starting points for areas of improvement of testicular models via bioengineering approaches.

- Cells are cultured in defined medium, which lacks the complexity and variety of nutrient support available in the *in vivo* environment.
- The medium supporting cells in culture is often static rather than flowing, so nutrients, secretions, and waste products remain in the medium with the cells rather than being transported away.
- Most cell cultures have less capacity for metabolizing added components.
- The normal three-dimensional architecture of the cells and tubules is lost.
- Endocrine and paracrine feedback loops present *in vivo* are disrupted or absent *in vitro*.
- There is no true blood-tubule barrier, although Sertoli-Sertoli and Sertoli-germ cell tight junctions do form.
- The correlation between the effects observed in non-human testicular cultures to humans is virtually unknown.

The last bullet leads into a critical topic: Should biomedical engineering efforts concentrate on developing *in vitro* models using human or non-human cells? The answer is perhaps not one or the other but that both are needed. Some labs are using non-human cells because the cells are readily available and *in vitro* results can be compared to well-understood *in vivo* effects in the same non-human species. Other laboratories are using human cells because understanding compound or disease effects in humans is the ultimate goal. There are innumerable reasons why either cell source is important in advancing our understanding of testicular toxicity; the choice will differ for each laboratory, and both are (currently) right. Due to the magnitude of this topic, it is only mentioned in this paper and will remain for future discussion.

5 Bioengineering approaches applicable to the design of improved testicular models

A bottom-up approach that allows for spatial and temporal control of the extra-cellular microenvironments has been achieved with bioengineered hepatic tissue. Evaluation of the methods employed for liver can shed light on possible approaches for testicular toxicity model building. Liver tissue engineering has

been implemented on the scale of 0.01 to <1 mm, where the degree of control or feature resolution is at the cellular level. Since liver is an organ with repeating units of lobules of 1-2 mm each, constructing one lobule or an acinus (part of a lobule with key hepatic function) should effectively reconstruct the basic unit and subsequent function of this organ. Such an approach is made possible by sophisticated micro-scale technologies (Ananthanarayanan et al., 2011; Bhatia et al., 1997), which facilitate the control of cell polarity and function. Intercellular tissue structure (bile canaliculi) recapitulate the *in vivo* environment and exposure and clearance of xenobiotics (Ananthanarayanan et al., 2011). It is believed that restoration of cell polarity and proper cell-cell communications, for instance, via gap junctions, are essential for functional restoration and maintenance over long culture periods in order to test tissue responses to test compounds and their metabolites (Patel et al., 2012).

There are a few microenvironmental variables that have been systematically modified to provide optimal support of cell function. These include cell-matrix and cell-cell interactions by modulating chemicals (Du et al., 2006), mechanical signaling (Nugraha et al., 2011) with innovative micro-bioreactors or chips (Toh et al., 2007), synthetic hydrogel and scaffold matrices (Choudhury et al., 2012), soluble factor presentations (Zhang et al., 2009a), and spatial and temporal shear stress control (Zhang et al., 2008) with controlled release devices and microfabricated ultrathin membranes with defined pores. These controls produce 3 primary classes of liver micro-tissue models: 1) 3-D hepatocyte models, 2) spatially-patterned co-culture models, and 3) perfusion cultures. The 3-D hepatocyte models include spheroids (Nugraha et al., 2011), a 3-D monolayer of polarized columnar epithelial cells with enhanced cell-cell contact areas (Du et al., 2006), or sandwich cultures where cells exhibit morphology reminiscent of *in vivo* cells (Farkas and Tannenbaum, 2005). The spatially patterned co-cultures have both heterotypic and homotypic cell-cell interactions that are spatially-controlled (Hui and Bhatia, 2007). The perfusion-cultures use bioreactors or microfluidic channels to mimic the microcirculation usually found *in vivo* (Sivaraman et al., 2005). These models of precisely controlled microenvironments have exhibited improved hepatocyte functions (up to 30x) over a longer period of time (up to 40 days in culture) than the standard 2-D cell cultures that contain one or two types of cells (Khetani and Bhatia, 2008). Efforts also have been made to improve mass transfer or drug access to 3-D cell cultures for pharmaceutical screening applications (Zhang et al., 2011).

For testicular toxicity models, several features of liver tissue engineering could be applicable including: 1) control of substrate mechanical and chemical cues to enable cells to "stand up" as a 3-D sheet in order to mimic the normal Sertoli morphology and better maintain function, 2) micropatterning of the Sertoli cells and germ cells to spatially and temporally control their interactions during differentiation, 3) incorporation of controlled release and perfusion features for soluble factors such as hormones, which normally are secreted by Leydig cells that trigger the germ cell differentiation processes, and 4) incorporation of small pumps to deliver appropriate micro-pulses of compound substances, thus simulating *in vivo* exposure to the test compound. For an introduction to cellular and tissue engineering see Yu and Rahim (2013).

The majority of existing liver models have been derived using rodent cells, whose isolation was reasonably standardized (Seglen, 1976). Such cellular sources were readily available at low prices and offered consistent performance. In addition, rodent cell models have enabled cross-species comparisons, and correlation between *in vitro* data and *in vivo* rodent data via use of sophisticated software packages such as SimCyp (Rostami-Hodjegan and Tucker, 2007).

However, translation of *in vitro* and *in vivo* animal data directly to humans is not always straightforward. Data from *in vitro* rodent cell models are not always consistent with the human cellular *in vitro* models or clinical data (Maier-Salamon et al., 2011). The majority of drug-testing models are assessed with a minimum of three lots of human hepatocytes, as human liver cells demonstrate larger batch to batch variations than do rodent cells (Tostoes et al., 2012). This situation is changing with the development of more consistent human cell sources such as the HepaRG cell line (Jennen et al., 2010) and humanized mice (Dorner et al., 2011) or other progenitor cells that can be expanded and differentiated either *in vivo* or *in vitro* with increasing cost effectiveness (Si-Tayeb et al., 2010).

Similarly, the development of testicular toxicity screening models would benefit from the development of an expandable source of Sertoli cells and germ/spermatogenic cells that can support spermatogenesis. It is entirely conceivable that such cells could be derived from induced pluripotent stem cells (iPS cells), provided the appropriate physical and hormonal environment is provided. For compound and chemical screening applications, it will be necessary to develop models that are scalable, robust, with consistent performance and highly sensitive to test compound-mediated effects. Examples of liver tissue engineering to map critical 3-D features into 2-D multi-well-based drug screening platforms (Du et al., 2006; Khetani and Bhatia, 2008; Toh et al., 2011) are readily applicable to the development of testicular toxicity testing applications.

Biomedical engineering principles that guide functional tissue engineering approaches for other tissues, such as bone, may provide insight for developing an *in vitro* testis model. For successful engineering of most tissues, it now is generally accepted that biomimetic material and mechanical stimuli are critical parameters. Mechanical stimuli *in vivo* predominantly include compression, fluid shear stress, and/or tensile strain that deform the cell membrane and activate surface membrane receptors (Jamney and Weitz, 2004). Cells also are exposed to material stimuli via the chemical, physical, and topographical properties of their substratum (Atala et al., 2008). Depending on the tissue, the material and mechanical environments to which cells are exposed can vary greatly. Cells residing in bone are exposed to very different physical stimuli than cells in cartilage, muscle, tendon, fat, liver, etc. These unique material and mechanical environments can guide cell proliferation, differentiation, and extracellular matrix production, and indeed are what help differentiate a bone cell from a testis cell.

In this context, common questions that arise in the mind of a biomedical engineer interested in designing a testicular toxicity

Tab. 3: Physiologic parameters of the testis

Parameter	Selected values	Comments	References
Temperature	32-34°C	Testicular temperature is maintained at 3 to 4ºC below body temperature	Brooks, 1973; Mieusset and Bujan, 1995
Intratesticular pressure	5.9 ±2.5 cm H_20	Capsule maintains intratesticular pressure	Moritoki et al., 2012
Testicular blood flow	7-12 cycles/min	Testicular microcirculation exhibits rhythmic cycles of blood flow known as vasomotion	Damber and Bergh, 1992; Collin et al., 2000; Lysiak et al., 2000
Oxygen tension	10.5-12.5 mm Hg	Low O_2 tension with cycles that correlate with testicular vasomotion	Setchell et al.,1994; Lysiak et al., 2000; Massie et al., 1969
Seminiferous tubule fluid flow rate	0.5-1 µl/h (0.017 µl/min)	Values are for the perfusion rate for a single seminiferous tubule. A perfusate flow rate at 0.14 µl/min did not adversely affect the morphology of the seminiferous epithelium in isolated tubules. However, high perfusate flow rates (≥0.42 µl/min) resulted in germ cell detachment from the seminiferous epithelium	Setchell et al., 1994; Fisher, 2002
Seminiferous tubule diameter	~200-350 µM	Some variation in the diameter with stage of spermatogenesis, largest diameter at stage VII and VIII of the spermatogenic cycle	Wing and Christensen, 1982; Sharpe, 1989
Seminiferous tubule lumen diameter	~100-200 µM	Diameter doubles in size at stages VII-VIII when elongate spermatids are about to be released	Wing and Christensen, 1982; Sharpe, 1989
Seminiferous tubule stiffness	No data	One study found that the stiffness of the hydrogel used in 2-D culture did not influence spermatogonial stem cell proliferation	Chu et al., 2009

model might include: 1) How do material properties change from the basal compartment to the apical surface of a seminiferous tubule, and what effects do these material properties have on the cells within these environments; 2) What are the properties of the testicular extracellular matrix, and how do material properties of current ECM substitutes (e.g., Matrigel®) compare to the native ECM; 3) What are the differing site-specific characteristics of the ECM; 4) What are the types and magnitudes of mechanical forces/stresses/strains during development and maturation of the fetal testis, and how can these be recapitulated *in vitro*; 5) How many of these characteristics will be necessary and/or useful to recapitulate in order to form a more perfect testicular toxicity model; 6) What are the differences in material and mechanical environments in normal versus abnormal testicular tissues; and, 7) Can computational models be implemented effectively as predictors to help drive the creation of *in vitro* models?

To date, few data have been published to address these questions (see Tab. 3). However, extensive studies have been performed investigating the effects of extracellular physical stimuli on cells from load-bearing tissues (muscle and bone; see review by McCullen et al., 2010). While such tissues obviously are different from testicular tissue, the basic premises related to successful tissue engineering still apply: characterize those measures in the tissue, and then recapitulate them *in vitro*. In particular, successful creation of a testicular toxicity model must incorporate not just an appropriate chemical environment but also the mechanical and material environments.

6 Physiological and morphological parameters that may be useful in designing prototypical testicular models

There are several decades of information on the anatomy and physiology of the testis that could be useful for engineering a testis *in vitro* such as Russell (1983). Table 3 below provides a brief selection of some physical characteristics of the testis/seminiferous tubules based on data collected from adult rats. The references provided are intended as a place to start to gather more information. It is outside the scope of this article to provide a comprehensive list of references. For detailed information on the anatomy and physiology of the testis for various species and the composition of fluids within the testis – venous blood, lymph fluid, interstitial fluid, seminiferous tubule fluid – see Setchell et al. (1994) and references therein.

Testicular blood flow is the main route for the transport of nutrients, secretory products, and oxygen to the testis, as well as for the elimination of waste products. Modulation of testicular blood flow and vascular permeability can influence testicular function. Laser Doppler flowmetry has shown that blood flow within the microvasculature of the testis shows rhythmic

variations of high and low flow known as vasomotion (Damber and Bergh, 1992; Collin et al., 2000). Vasomotion has been described in several species (Collin et al., 2000). Vasomotion influences the formation and resorption of interstitial fluid, and thus changes in vasomotion affect the volume and composition of the interstitial fluid (Damber and Bergh, 1992). Hypoxia and hormones influence vasomotion, and testosterone is one of the key factors that control vasomotion and blood flow (Lysiak et al., 2000; Collin et al., 1993, 2000). Short-term reductions in blood flow can result in testicular damage (Bergh et al., 2001). Oxygen tension within the testis is low, and spermatogenesis appears to be adapted for a hypoxic environment. Interestingly, the interstitial oxygen tension also appears to show rhythmic variations with the same frequency as that observed for vasomotion (Lysiak et al., 2000). While any successful *in vitro* model of spermatogenesis likely will be low-oxygen, the importance of low-amplitude cycles reminiscent of vasomotion currently is unknown.

Detailed morphometry studies on the surface-to-volume relationships and cellular ratios between Sertoli cells and the developing germ cells have been performed in the adult rat (see Wing and Christensen, 1982; Russell et al., 1983; Weber et al., 1983; Renato de Franca, 1993; Russell and Renato de Franca, 1995) and references therein. Such information can be combined with the recent advance in multi-scale tissue engineering technologies on kidney, liver, embryo, and other soft tissues (see Ananthanarayanan et al., 2011; Toh et al., 2011, Wen et al., 2008) to update the previously discussed co-culture models with improved controls of the microenvironment (cell-substrate and cell-cell interaction, and spatiotemporal presentation of soluble factors) such that spermatogenesis might consistently occur *in vitro*, yet be simple and robust enough for drug screening. It is conceivable that chips or multiwell bioreactors can be engineered that enable Sertoli cells to interact with germ cells in the presence of properly presented cytokines, either secreted by Leydig cells co-cultured in adjacent compartments or control-released locally (Zhang et al., 2009a,b). The more complex perfusion models also can simulate the rhythmic variation of blood flow and oxygen tension during critical stages of spermatogenesis in culture, if necessary, and then be removed to screenable static culture multiwell platforms in drug screening applications (Zhang et al., 2011).

One of the key considerations when developing 3-D testicular cell culture systems is the type and organization of the scaffold to be used. The scaffold must present to the cells the appropriate chemical and mechanical cues often rendered by the ECM. Ideally, a matrix that reflects the composition and organization of the ECM that exists *in vivo* probably would result in a more *in vivo*-like culture system. Chemically, the basement membrane of the seminiferous tubule acts as the extracellular matrix, and a variety of components have been identified in the basement membrane, including collagen IV, laminin, entactin, heparin sulfate proteoglycans, and fibronectin (see Siu and Cheng, 2004 and references therein). Mechanical properties of the environment are related to topography and substrate rigidity (typically kPa range) that are rendered by neighboring cells and ECM (Chang et al., 2009) that can be provided by various hydrogels. In the testis, ECM does not block the paracrine signals between the tubules and the interstitium. Hydrogels often can block such signals. Thus, other soft and macroporous scaffold materials (Yue et al., 2010; Nugraha et al., 2011) that have suitable chemical and mechanical properties to facilitate cell-cell interaction can be used.

7 Available biochemical markers that can be used to monitor cellular health of *in vitro* testicular systems

Several recent articles have described a variety of molecular markers that have been used to characterize the cell types as the cells grow and differentiate in *in vitro* culture systems (Abu Elhija et al., 2012; Gassei et al., 2008; Legendre et al., 2010; Stukenborg et al., 2008). Various methods have been used to evaluate expression of cell markers, including reverse-transcriptase RT-PCR, flow cytometry, and immunohistochemistry. Some examples of cell markers that have been used to identify cells in testicular cultures are summarized in Table 4.

In essence, these markers act as cell identifiers, reporting on which cell populations exist in the cultures. Table 4 summarizes a selection of markers from various laboratories that have been used recently and the associated references to studies in which they were used. These are examples, not a definitive list, nor an endorsement for use. Selection of markers to identify testicular cells used in culture would be based on the needs of the investigator to determine which markers would be most applicable to their use based on cell types they are culturing and access to the appropriate tools to detect the markers.

In addition, functional parameters can be measured, both *in vivo* and *in vitro*. One of the key characteristics of the testis is the synthesis of testosterone by Leydig cells. For cultures involving Leydig cells, culture media can be sampled and testosterone production can be measured in stimulated (e.g., LH or dibutyryl cAMP) and unstimulated conditions (Midzak et al., 2011). Germ cells are intimately attached to Sertoli cells by adhesion junctions (Russell and Peterson, 1985). Various Sertoli cell toxicants such as 2, 5-hexanedione, 1,3-dinitrobenzene, and phthalates induce detachment of germ cells from the seminiferous epithelium *in vivo* (see Pop-Off section, above, and Boekelheide et al., 2005 for references). Sertoli cells produce lactate and pyruvate *in vitro*, and production can be stimulated by FSH (Jutte et al., 1983). Studies have demonstrated that dose-related increases in pyruvate and/or lactate secretion are induced by treatment with the Sertoli cell toxicants (1,3-dinitrobenzene, mono-(2-ethylhexyl) phthalate, and dinitrotoluene) but not with the germ cell toxicant ethylene glycol monomethyl ether (Williams and Foster, 1988; Reader and Foster, 1990). Exactly how measures of these intermediates, which respond the same way to both physiologic stimuli and toxicologic stimuli, would be used and interpreted is quite uncertain.

Sertoli cells produce seminiferous tubule fluid (STF) to maintain the appropriate nutritional and hormonal environment to support spermatogenesis and to transport sperm to the epididymis. Seminiferous tubule fluid secretion can be measured *in*

Tab. 4: Examples of markers to identify testicular cells used in culture

Cell Type	Marker	References
Sertoli cell – immature	Cytokeratin 18; Gata-4; antimullerian hormone	Gassei et al., 2008; Gassei et al., 2010
Sertoli cell – mature	Transferrin; FSH receptor; aromatase (cytochrome P450 19a1); androgen binding protein; sulphated glycoproteins; inhibin α	Gassei et al., 2008; Abu Elhija et al., 2012; Walther et al., 1996
Spermatogonia	Nanog; octamer-binding transcription factor 4 (Oct-4); stem cell growth factor receptor (c-Kit); GFR-α-1 (glial cell line-derived neurotrophic factor receptor); α-6-integrin; CD9; Notch-1	Abu Elhija et al., 2012; Stukenborg et al., 2008; Legendre et al., 2010; von Schönfeldt et al., 2004
Spermatocytes	Prohibitin; synaptonemal complex protein 3 (Scp-3); Boule	Stukenborg et al., 2008
Round spermatids	Crem-1; lactate dehydrogenase; protamine 2; SP-10 (testis-specific acrosomal protein)	Abu Elhija et al., 2012; Stukenborg et al., 2008
Elongating spermatids	Protamine-1; acrosin	Abu Elhija et al., 2012
Peritubular myoid cells	α smooth muscle actin; alkaline phosphatase	Gassei et al 2008; Abu Elhija et al., 2012; Legendre et al., 2010
Leydig cells	LH receptor; cytochrome P450 side chain cleavage enzyme (Cyp11a1); 3β-hydroxysteroid dehydrogenase; 17α-hydroxylase/17,20 lyase (Cyp17a1); steroidogenic acute regulatory protein (StAR)	Abu Elhija et al., 2012; Gassei et al., 2008; Ge et al., 2006
Macrophages	CD11b; EMR1-F4/80	Abu Elhija et al., 2012; Itoh et al., 1995

Tab. 5: Functional parameters that can be assessed *in vitro*

Parameter	Functionality	Selected references
Testosterone production	*In vitro* testosterone production is a marker of viable Leydig cells with normal physiological function	Midzak et al., 2011
Germ cell detachment	Germ cell detachment has been demonstrated to be a sensitive marker of altered Sertoli cell function in Sertoli-germ cell co-cultures *in vitro*	Reader and Foster, 1990; Yao et al., 2010
Lactate and pyruvate production	Increases in lactate and pyruvate production have been demonstrated to be sensitive markers of altered Sertoli cell function in Sertoli cell and Sertoli-germ cell co-cultures *in vitro*	Williams and Foster, 1988; Reader and Foster, 1990
Seminiferous tubule fluid production	*In vitro* decreased seminiferous tubule fluid production has been demonstrated to be a marker of altered Sertoli cell function	Richburg et al., 1994

vivo by ligating the efferent ducts. Exposure to Sertoli cell toxicants has been shown to reduce STF secretion *in vivo* (Johnson et al., 1991; Gray and Gangolli, 1986). Secretion of STF also has been quantified *in vitro* using the rate of transport of microinjected oil droplets in the lumen of isolated rat seminiferous tubules (Richburg et al., 1994). Similar to the *in vivo* results, the rate of transport of the luminal oil droplets was decreased in seminiferous tubules from rats exposed previously to 2,5-hexanedione (Richburg et al., 1994). Some examples of the types of parameters that can be measured are summarized in Table 5.

However, we need to remember that any *in vitro* model of toxicity will only be able to report on mechanisms that occur in the model. So a model of spermatogenesis, no matter how ingenious or effective, will not be able to tell us about toxicity to the pampiniform plexus, or the vascular system, or fluid flow to the epididymis, or effects on sperm glycolysis, or impact on fertilization. As long as we remember that these are inappropriate questions for a spermatogenesis model and recall the limitations of the answers, all will be well.

8 Discussion

While nonclinical safety assessment is required for the registration of pharmaceuticals and new and existing chemicals, current *in vivo* approaches to evaluate male reproductive toxicity

use large numbers of animals and are costly and time-consuming. Although numerous *ex vivo* and *in vivo* testicular toxicity models have been developed and improved over the past several decades, the complex interaction of Sertoli cells, germ cells, and Leydig cells and the dynamic processes of spermatogenesis, which are tightly controlled by intercellular signaling pathways, are not well modeled in currently available *in vitro* testing platforms.

Advances in biomedical engineering provide a possible solution to the challenges of sustained culture of adult-derived cells. Information from the historical literature on the morphology and physiology of the testis can provide a good starting point for tissue engineers to build small multicellular units that could be grown in three-dimensional scaffolds that approximate *in vivo* tissue structure and enable maintenance and "cross-talk" between mixed cell types. Human testicular tissue obtained via commercial suppliers, traditionally, has been sourced from donors of variable ages, environmental influences, and health status. However, it is conceivable that, as the need for testicular toxicity models expands, suppliers may be able to identify and offer more consistent tissue sources, similar to the evolution of 3-dimensional skin assay kits for genotoxicity testing (Kirsch-Volders et al., 2011; Aardema et al., 2010). Alternatively, novel sources of cells such as stem cells or induced pluripotent stem cells (iPS) could enable creation of reproducible sources of human testicular cell types.

Despite the challenges, development of 3-D *in vitro* models derived from human tissue is considered by many as an ultimate goal. Recently, the US Defense Advanced Research Projects Agency (DARPA) and US FDA announced a call for grant proposals that would develop *in vitro* constructs that can "reproduce relatively authentic human tissue and organ physiology in an *in vitro* organ environment" (DARPA, 2011). Such *in vitro* models would be expected to produce human-relevant, *in vivo* predictive responses to toxic agents. Even with the development of human cell-based systems, a great challenge following the generation of data from human cell-based *in vitro* systems will be the interpretation of the risk and relevance of this information in the context of *in vivo* data. For instance, if animal *in vivo* data suggest toxicity but the human cellular *in vitro* systems indicate a lack of toxicity, would such an agent be assumed to have a favorable human safety profile? In parallel to the development of alternative models, the applicability domain of each testing approach must be defined in order to design *in vitro*, *ex vivo*, or *in silico* assay combinations that can reduce and refine *in vivo* testing. Along such lines, recent topics for funding, such as the European Commission FP7 Cooperation Work Programme Health 2013[9], seek collaborative projects that will integrate computational and systems biology approaches with *in vitro* data.

It is hoped that the information disseminated at this workshop serves as useful background for investigators seeking to design improved *in vitro* testicular models. Based on near-term applications for environmental chemicals and pharmaceutical screening, recent bioengineering advances with liver and bone, and funding interest among government agencies, development of predictive *in vitro* testicular platforms is an attractive near and long-term goal for the scientific community.

[9] http://cordis.europa.eu/fp7/health/

References

Aardema, M. J., Barnett, B. C., Khambatta, Z., et al. (2010). International prevalidation studies of the EpiDerm 3D human reconstructed skin micronucleus (RSMN) assay: transferability and reproducibility. *Mutat Res 30*, 123-131.

Abu Elhija, M., Lunenfeld, E., Sclatt, S., and Huleihel, M. (2012). Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian J Androl 14*, 285-293.

Adhikari, N., Sinha, N., and Saxena, D. K. (2000). Effect of lead on Sertoli-germ cell coculture of rat. *Toxicol Lett 116*, 45-49.

Agarwal, D. K., Eustis, S., Lamb, J. C., et al. (1986). Effects of Di(2-ethylhexyl) phthalate on the gonadal pathophysiology, sperm morphology, and reproductive performance of male rats. *Environ Health Perspect 65*, 343-350.

Ailenberg, M., Tung, P. S., Pelletier, M., and Fritz, I. B. (1988). Modulation of Sertoli cell functions in the two-chamber assembly by peritubular cells and extracellular matrix. *Endocrinol 122*, 2604-2612.

Allenby, G., Foster, P. M. D., and Sharpe, R. M. (1991a). Evidence that secretion of immunoactive inhibin by seminiferous tubules from the adult rat testis is regulated by specific germ cell types: correlation between in vivo and in vitro studies. *Endocrinol 128*, 467-476.

Allenby, G., Foster, P. M. D., and Sharpe, R. M. (1991b). Evaluation of changes in the secretion of immunoactive inhibin by adult rat seminiferous tubules in vitro as an indicator of early toxicant action on spermatogenesis. *Fund Appl Toxicol 16*, 710-724.

Ananthanarayanan, A., Narmada, B. C., Mo, X., et al. (2011). Purpose-driven biomaterials research in liver-tissue engineering. *Trends Biotechnol 29*, 110-118.

Apostoli, P. A., Kiss, P., Porru, S., et al. (1998). Male reproductive toxicity of lead in animals and humans. *Occup Environ Med 55*, 364-374.

Atala, A., Lanza, R., Thomson, J., and Nerem, R. (2008). *Principles of Regenerative Medicine*. 1st edition. Boston, MD, USA: Elsevier.

Barton, H. A., Pastoor, T. P., Baetcke, K., et al. (2006). The acquisition and application of absorption, distribution, metabolism, and excretion (ADME) data in agricultural. *Crit Rev Toxicol 36*, 9-35.

Bergh, A., Collin, O., and Lissbrant, E. (2001). Effects of acute graded reductions in testicular blood flow on testicular morphology in the adult rat. *Biol Reprod 64*, 13-20.

Bhatia, S. N., Yarmush, M. L., and Toner, M. (1997). Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. *J Biomed Mater Res 34*, 189-199.

Bilinska, B. (1989). Interaction between Leydig and Sertoli cells in vitro. *Cytobios 60*, 115-126.

Boekelheide, K., Johnson, K. L., and Richburgh, J. H. (2005). Sertoli cell toxicants. In M. K. Skinner and M. D. Griswold (eds), *Sertoli Cell Biology*. San Diego, CA, USA and London, UK: Elsevier Academic Press.

Bremer, S., Pellizzer, C., Hoffmann, S., et al. (2007). The development of new concepts for assessing reproductive toxicity applicable to large scale toxicological programmes. *Curr Pharm Des 13,* 3047-3058.

Brooks, D. E. (1973). Epididymal and testicular temperature in the unrestrained conscious rat. *J Reprod Fert 35*, 157-160.

Brun, H. P., Leonard, J. F., Moronvalle, V., et al. (1991). Pig Leydig cell culture: a useful in vitro test for evaluating the testicular toxicity of compounds. *Tox Appl Pharmacol 108*, 307-320.

Byers, S. W., Hadley, M. A., Djakiew, D., and Dym, M. (1986). Growth and characterization of polarized monolayers of epididymal epithelial cells and Sertoli cells in dual environment culture chambers. *J Androl 7*, 59-68.

Carmichael, N. G., Barton, H. A., Boobis, A. R., et al. (2006). Agricultural chemical safety assessment: A multisector approach to the modernization of human safety requirements. *Crit Rev Toxicol 36*, 1-7.

Cave, D. A. and Foster, P. M. (1990). Modulation of m-dinitrobenzene and m-nitrosonitrobenzene toxicity in rat Sertoli-germ cell cocultures. *Fundam Appl Toxicol 14*, 199-207.

Chang, H. K., Park, Y. J., Koh, H., et al. (2009). Hepatic fibrosis scan for liver stiffness score measurement: a useful preendoscopic screening test for the detection of varices in postoperative patients with biliary atresia. *J Pediatr Gastroenterol Nutr 49*, 323-328.

Chapin, R. E., Gray, T. J., Phelps, J. L., and Dutton, S. L. (1988). The effects of mono-(2-ethylhexyl)-phthalate on rat Sertoli cell-enriched primary cultures. *Toxicol Appl Pharmacol 92*, 467-479.

Chapin, R. E. and Phelps, J. (1990). Recent advances in testicular cell culture: implications for toxicology. *Toxicol In Vitro 4*, 543-559.

Chapin, R. E., Phelps, J. L., Somkuti, S. G., et al. (1990). The interaction of Sertoli and Leydig cells in the testicular toxicity of tri-o-cresyl phosphate. *Toxicol Appl Pharmacol 104*, 483-495.

Chapin, R. E., Phelps, J. L., Burka, L. T., et al. (1991). The effects of tri-o-cresyl phosphate and metabolites on rat Sertoli cell function in primary culture. *Tox Appl Pharmacol 108*, 194-204.

Chauvigné, F., Plummer, S., Lesné, L., et al. (2011). Mono-(2-ethylhexyl) phthalate directly alters the expression of Leydig cell genes and CYP17 lyase activity in cultured rat fetal testis. *PLoS One 6*, e17172.

Choudhury, D., van Noort, D., Iliescu, C., et al. (2012). Fish and Chips: a microfluidic perfusion platform for monitoring zebrafish development. *Lab Chip 12*, 892-900.

Chu, C., Schmidt, J. J., Carnes, K., et al. (2009). Three-dimensional synthetic niche components to control germ cell proliferation. *Tissue Eng Part A 15*, 255-262.

Chubb, C. and Ewing, L. L. (1981). Steroid secretion by sexually immature rat and rabbit testes perfused in vitro. *Endocrinol 109*, 1999-2003.

Chung, N. P. Y. and Cheng, C. Y. (2001). Is cadmium chloride-induced inter-Sertoli tight junction permeability barrier disruption a suitable in vitro model to study the events of junction disassembly during spermatogenesis in the rat testis? *Endocrinol 142*, 1878-1888.

Coffey, J. C., French, F. S., and Neyfeh, S. N. (1971). Metabolism of progesterone by rat testicular homogenates. IV. Further studies of testosterone formation in immature rat testis in vitro. *Endocrinol 89*, 865-872.

Collin, O., Bergh, A., Damber, J. E., and Widmark, A. (1993). Control of testicular vasomotion by testosterone and tubular dactors in rats. *J Reprod Fertil 97*, 115-121.

Collin, O., Zupp, J. L., and Setchell, B. P. (2000). Testicular vasomotion in different mammals. *Asian J Androl 2*, 297-300.

Creasy, D. M. and Foster, P. M. (1984). The morphological development of glycol ether-induced testicular atrophy in the rat. *Exp Mol Pathol 40*, 169-176.

Creasy, D. M., Beech, L. M., and Gray, T. J. (1988). Effects of mono-(2-ethylhexyl) phthalate and mono-n-pentyl phthalate on the ultrastructural morphology of rat Sertoli cells in Sertoli/germ cell co-cultures: Correlation with the in vivo effects of di-n-pentyl phthalate. *Toxicol In Vitro 2*, 83-95.

Creasy, D. M. (2002). Histopathology of the male reproductive system II: interpretation. *Curr Protoc Toxicol 16.4*.

Creasy, D. M. and Foster, P. M. D. (2002). Male reproductive system. In W. M. Haschek, C. G. Rousseaux, and M. A. Wallig (eds), *Handbook of Toxicologic Pathology*. 2nd edition. Vol. 2. Academic Press Inc.

Cukierman, E., Pankov, R., Stevens, D. R., and Yamada, K. M. (2001). Taking cell-matrix adhesions to the third dimension. *Science 294*, 1708-1712.

Damber, J. E. and Bergh, A. (1992). Testicular microcirculation – a forgotten essential in andrology? *Inter J Androl 15*, 285-292

Danadevi, K., Rozati, R., Reddy, P. P., and Grover, P. (2003). Semen quality of Indian welders occupationally exposed to nickel and chromium. *Reprod Toxicol 17*, 451-456.

Dang, Z. C., Rorije, E., Esch, T. H., et al. (2009). Retrospective analysis of relative parameter sensitivity in multi-generation reproductive toxicity studies. *Reprod Toxicol 28*, 196-202.

Dann, C. T., Alvarado, A. L., Molyneux, L. A., et al. (2008). Spermatogoinial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells 26*, 2928-2937.

DARPA - Defense Advanced Research Projects Agency (2011). Broad Agency Announcement: Microphysiological Systems. DSO, DARPA-BAA-11-73.

De Krester, D. M. and Kerr, J. B. (1988). The cytology of the testis. In E. Knobil and J. Neill (eds). *The Physiology of Reproduction* (837-932). New York, USA: Raven Press.

Dix, D. J. and Kavlock, R. J. (2011). Activity profiles of 309 ToxCast™ chemicals evaluated across 292 biochemical targets. *Toxicology 282*, 1-15.

Djakiew, D., Hadley, M. A., Byers, S. W., and Dym, M. (1986). Transferrin-mediated transcellular transport of 59Fe across confluent epithelial sheets of Sertoli cells grown in bicameral cell culture chambers. *J Androl 7*, 355-366.

Dorner, M., Horwitz, J. A., Robbins, J. B., et al. (2011). A genetically humanized mouse model for hepatitis C virus infection. *Nature 474*, 208-211.

Du, Y., Chia, S. M., Han, R., et al. (2006). 3D hepatocyte monolayer on hybrid RGD/galactose substratum. *Biomaterials 27*, 5669-80.

Dufau, M. L. (1988). Endocrine regulation and communicating functions of the Leydig cell. *Annu Rev Physiol 50*, 483-508.

Elkin, N. D., Piner, J. A., and Sharpe, R. M. (2010). Toxicant-induced leakage of germ cell-specific proteins from seminiferous tubules in the rat: relationship to blood-testis barrier integrity and prospects for biomonitoring. *Toxicol Sci 117,* 439-448.

Ernst, E. (1990). Testicular toxicity following short-term exposure to tri- and hexavalent chromium: an experimental study in the rat. *Toxicol Lett 51*, 269-275.

Eskenazi, E. and Kimmel, G. (1995). Workshop on perinatal exposure to dioxin-like compounds. II. Reproductive effects. *Environ Health Perspect 103*, 143-145.

European Commission (2007). Regulation (EC) No. 1907/2006 of the European Parliament and the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm

Ewing, L. L. and Zirkin, B. (1983). Leydig cell structure and steroidogenic function. *Recent Prog Horm Res 39*, 599-635.

Farkas, D. and Tannenbaum, S. R. (2005). Characterization of chemically induced hepatotoxicity in collagen sandwiches of rat hepatocytes. *Toxicol Sci 85*, 927-934.

Ficher, M. and Steinberger, E. (1971). In vitro progesterone metabolism by rat testicular tissue at different stages of development. *Acta Endocrinol 68*, 285-292.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., et al. (2004). Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. *Reprod Toxicol 18*, 413-421.

Fisher, D. (2002). New light shed on fluid formation in the seminiferous tubules of rats. *J Physiol 542*, 445-452.

Foster, P. M., Creasy, D. M., Foster, J. R., et al. (1983). Testicular toxicity of ethylene glycol monomethyl and monoethyl ethers in the rat. *Toxicol Appl Pharmacol 69*, 385-399.

Foster, P. M., Creasy, D. M., Foster, J. R., and Gray, T. J. (1984). Testicular toxicity produced by ethylene glycol monomethyl and monoethyl ethers in the rat. *Environ Health Perspect 57*, 207-217.

Foster, P. M., Lloyd, S. C., and Prout, M. S. (1987). Toxicity and metabolism of 1,3-dinitrobenzene in rat testicular cell cultures. *Toxicol In Vitro 1*, 31-37.

Fritz, I. B., Parvinen, M., Karmally, K., and Lecroix, M. (1981). Preferential production of testicular plasminogen activator by Sertoli cells in discrete portions (Stages VII and VIII) of the seminiferous tubule. *Ann NY Acad Sci 383*, 447-448.

Gassei, K., Ehmcke, J., and Schlatt, S. (2008). Initiation of testicular tubulogenesis controlled by neurotrophoc tyrosine receptor kinases in a three-dimensional Sertoli cell aggregation assay. *Reproduction 136*, 459-469.

Gassei, K., Ehmcke, J., Wood, M. A., et al. (2010). Immature rat seminiferous tubules reconstructed in vitro express markers of Sertoli cell maturation after xenografting into nude mouse hosts. *Mol Hum Reprod 16*, 97-110.

Ge, R.-S., Dong, Q., Sottas, C. M., et al. (2006). In search of rat stem Leydig cells: Identification, isolation, and lineage-specific development. *Proc Natl Acad Sci 103*, 2719-2724.

Geoffroy-Siraudin, C., Perrard, M.-H., Chaspoul, F., et al. (2010). Validation of a rat seminiferous tubule culture model as a suitable system for studying toxicant impact on meiosis effect of hexavalent chromium. *Toxicol Sci 116*, 286-296.

Goldman, A. S. and Klingle, D. A. (1974). Developmental defects of testicular morphology and steroidogenesis in the male rat pseudohermaphrodite and response to testosterone and dihydrotestosterone. *Endocrinol 94*, 1-16.

Gordeladze, J. O., Parvinen, M., Clausen, O. P., and Hansson, V. (1982). Stage dependent variation in MN2+ sensitive adenylyl cyclias (AC) activity in spermatids and FSH-sensitive AC in Sertoli cells. *Arch Androl 8*, 43-51.

Gray, T. J. and Beamand, J. A. (1984). Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. *Food Chem Toxicol 22*, 123-131.

Gray, T. J. B. (1986). Testicular toxicity in vitro: Sertoli-germ cell co-cultures as a model system. *Food Chem Toxicol 24*, 601-605.

Gray, T. J. B. and Gangolli, G. (1986). Aspects of the testicular toxicity of phthalate esters. *Environ Health Perspect 65*, 229-235.

Gregotti, C., Di Nucci, A., Costa, L. G., et al. (1992). Effects of thallium on primary cultures of testicular cells. *J Toxicol Environ Health 36*, 59-69.

Grima, J. and Cheng, C. Y. (2000). Testin induction: the role of cyclic 3',5'- adenosine monophosphate/protein kinase A signaling in the regulation of basal and lonidamine-induced testin expression in rat Sertoli cells. *Biol Reprod 63*, 1648-1660.

Grootegoed, J. A., Krüger-Sewnarain, B. C., Jutte, N. H. P. M., et al. (1982). Fucosylation of glycoproteins in rat spermatocytes and spermatids. *Gamete Res 5*, 303-315.

Gye, M. C. (2003). Changes in the expression of claudins and transepithelial electrical resistance of mouse Sertoli cells by Leydig cell coculture. *Int J Androl 26*, 271-278.

Hadley, M. A., Byers, S. W., Suarez-Quian, C. A., et al. (1985). Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. *J Cell Biol 101*, 1511-1522.

Hadley, M. A., Byers, S. W., Suarez-Quian, C. A., et al. (1988). In vitro models of differentiated Sertoli cell structure and function. *In vitro Cell Develop Biol 24*, 550-557.

Hall, P. F. (1988). Testicular steroid synthesis: organization and regulation. In E. Knobil and J. Neill (eds). *The Physiology of Reproduction* (975-998). New York, NY, USA: Raven Press.

Hardin, B. D. (1983). Reproductive toxicity of glycol ethers. *Toxicology 27*, 91-102.

Hardy, M. P., Zirkin, B. R., and Ewing, L. L. (1989). Kinetic studies on the development of the adult population of Leydig cells in testes of pubertal rats. *Endocrinol 124*, 762-770.

Hardy, M. P., Kelce, W. R., Klinefelter, G. R., and Ewing, L. L. (1990). Differentiation of Leydig cell precursors In vitro: A role for androgen. *Endocrinol 127*, 488-490.

Hareng, L., Pellizzer, C., Bremer, S., et al. (2005). The Integrated Project ReProTect: A novel approach in reproductive toxicity hazard assessment. *Reprod Toxicol 20*, 441-452.

Hartung, T. and McBride, M. (2011). Food for thought … on mapping the human toxome. *ALTEX 28*, 83-93.

Hartung, T. and Rovida, C. (2009). Chemical regulators have overreached. *Nature 460*, 1080-1081.

Hartung, T., Luechtefeld, T., Maertens, A., and Kleensang, A. (2013). Integrated testing strategies for safety assessments. *ALTEX 30*, 3-18.

Hauser, R., Williams, P., Altshul, L., and Calafat, A. M. (2005). Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. *Environ Health Perspect 113*, 425-430.

Hauser, R., Meeker, J. D., Singh, N., et al. (2007). DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod 22*, 688-695.

Heim, C., Minniear, K., and Dann, K. T. (2011). Imatinib has deleterious effects on differentiating spermatogonia while sparing spermatogonial stem cell self renewal. *Reprod Toxicol 31*, 454-463.

Hellstrom, W. J. G., Overstreet, J. W., Yu, A., et al. (2003). Tadalafil has no detrimental effect on human spermatogenesis or reproductive hormones. *J Urol 170*, 887-891.

Hellstrom, W. J. G., Gittelman, M., Jarow, J., et al. (2008). An evaluation of semen characteristics in men 45 years of age or older after daily dosing with tadalafil 20mg: results of a multicenter, randomized, double-blind, placebo-controlled, 9-month study. *Eur Urol 53*, 1058-1065.

Holmes, S. D., Bucci, L. R., Lipshultz, L. I., and Smith, R. G. (1983). Transferrin binds specifically to pachytene spermatocytes. *Endocrinol 113*, 1916-1918.

Hui, E. E. and Bhatia, S. N. (2007). Micromechanical control of cell-cell interactions. *Proc Natl Acad Sci 104*, 5722-5726.

Itoh, M., De Rooij, D. G., Jansen, A., and Drexhage, A. M. (1995). Phenotypical heterogeneity of testicular macrophages/dendritic cells in normal adult mice: an immunohistochemical study. *J Repro Immunol 28*, 217-232.

Jamney, P. A. and Weitz, D. A. (2004). Dealing with mechanics: mechanisms of force transduction in cells. *Trends Biochem Sci 29*, 364-370.

Janecki, A. and Steinberger, A. (1986). Polarized Sertoli cell function in a new two-compartment culture system. *J Androl 7*, 69-71.

Janecki, A., Jakubowiak, A., and Steinberger, A. (1990). Vectorial secretion of inhibin by immature rat Sertoli cells in vitro: reexamination of previous results. *Endocrinol 127*, 1896-1903.

Janecki, A., Jakubowiak, A., and Steinberger, A. (1992). Effect of cadmium chloride on transepithelial electrical resistance of Sertoli cell monolayers in two-compartment cultures – A new model for toxicological investigations of the "blood-testis" barrier in vitro. *Toxicol Appl Pharmacol 112*, 51-57.

Jennen, D. G., Magkoufopoulou, C., Ketelslegers, H. B., et al. (2010). Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol Sci 115*, 66-79.

Johnson, K. J., Hall, E. S., and Boekelheide, K. (1991). 2,5-Hexanedione exposure alters the rat Sertoli cell cytoskeleton. I. Microtubules and seminiferous tubule fluid secretion. *Toxicol Appl Pharmacol 111*, 432-442.

Jutte, N. H., Jansen, R., Grootegoed, J. A., et al. (1983). FSH stimulation of the production of pyruvate and lactate by rat Sertoli cells may be involved in hormonal regulation of spermatogenesis. *J Reprod Fertil 68*, 219-226.

Jutte, N. H., Jansen, R., Grootegoed, J. A., et al. (1985). Protein synthesis by isolated pachytene spermatocytes in the absence of Sertoli cells. *J Exp Zool 233*, 285-290.

Kaitu-u-Lino, T. J., Sluka, P., Foo, C. F. H., and Stanton, P. G. (2007). Claudin-11 expression and localization is regulated by androgens in rat Sertoli cells in vitro. *Reproduction 133*, 1169-1179.

Kerr, J. B. and Knell, C. M. (1988). The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. *Development 103*, 535-544.

Khetani, S. R. and Bhatia, S. N. (2008). Microscale culture of human liver cells for drug development. *Nat Biotechnol 26*, 120-126.

Kirsch-Volders, M., Decordier, I., Elhajouji, A., et al. (2011). In vitro genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models. *Mutagenesis 26*, 177-184.

Klinefelter, G. R. and Ewing, L. L. (1989). Maintenance of testosterone production by purified adult rat Leydig cells for 3 days in vitro. *In Vitro Cell Dev Biol 25*, 283-288.

Kotaja, N., Kimmins, S., Brancorsini, S., et al. (2004). Preparation, isolation and characterization of sperm-specific spermatogenic cells for cellular and molecular analysis. *Nature Methods 1*, 249-254.

Kotula-Balak, M., Pochec, E., Hejmej, A., et al. (2011). Octylphenol affects morphology and steroidogenesis in mouse tumor Leydig cells. *Toxicol In Vitro 25*, 1018-1026.

Knudsen, T. B., Houck, K. A., Sipes, N. S., et al. (2011). Activity profile of 309 TOXCAST TM chemicals evaluated across 292 biochemical targets. *Toxicology 282*, 1-15.

Ku, W. W., Shih, L. M., and Chapin, R. E. (1993). The effects of boric acid (BA) on testicular cells in culture. *Reprod Toxicol 7*, 321-331.

Ku, W. W. and Chapin, R. E. (1994). Spermatocyte toxicity of 2-methoxyethanol in vivo and in vitro: requirements for an intact seminiferous tubule structure for germ cell degeneration. *Toxicol In Vitro 8*, 1191-1202.

Lamb, J. C., 4th and Chapin, R. E. (1993). Testicular and germ cell toxicity: in vitro approaches. *Reprod Toxicol 7*, 17-22.

LeBlond, C. P. and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann NY*

Acad Sci 55, 548-573.

LeCroix, M., Parvinen, M., and Fritz, I. B. (1981). Localization of testicular plasminogen activator in discrete portions (Stages VII and VIII) of the seminiferous tubules. *Biol Reprod 25*, 143-146.

Legendre, A., Froment, P., Desmots, S., et al. (2010). An engineered 3D blood-testis barrier model for the assessment of reproductive toxicity potential. *Biomaterials 31*, 4492-4505.

Lejeune, H., Sanchez, P., and Saez, J. M. (1998). Enhancement of long-term testosterone secretion and steroidogenic enzyme expression in human Leydig cells by co-culture with human Sertoli cell-enriched preparations. *Int J Andro 21*, 129-140.

Li, L. H., Jester, W. F., Jr., and Orth, J. M. (1998). Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol 153*, 258-265.

Li, M. W., Mruk, D. D., Lee, W. M., and Cheng, C. Y. (2009). Disruption of the blood-testis barrier integrity by bisphenol A in vitro: Is this a suitable model for studying blood-testis barrier dynamics? *Int J Biochem Cell Biol 41*, 2302-2314.

Lilly (2010). Cialis package insert, accessed on Dec, 2012. http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021368s017lbl.pdf

Lysiak, J. J., Nguyen, Q. A. T., and Turner, T. T. (2000). Fluctuations in rat testicular interstitial oxygen tensions are linked to testicular vasomotion: persistence after repair of torsion. *Biol Reprod 63*, 1383-1389.

Maier-Salamon, A., Bohmdorfer, M., Thalhammer, T., et al. (2011). Hepatic glucuronidation of resveratrol: interspecies comparison of enzyme kinetic profiles in human, mouse, rat, and dog. *Drug Metab Pharmacokinet 26*, 364-373.

Mann, P. C. (1997). Selected lesions of dioxin in laboratory rodents. *Tox Path 25*, 72-79.

Marcon, L., Zhang, X., Hales, B. F., et al. (2010). Development of a short-term fluorescence-based assay to assess the toxicity of anticancer drugs on rat stem/progenitor spermatogonia in vitro. *Biol Reprod 83*, 228-237.

Martin, M. T., Mendez, E., Corum, D. G., et al. (2009). Profiling the reproductive toxicity of chemicals from multigeneration studies in the toxicity reference database. *Toxicol Sci 110*, 181-190.

Massie, E. D., Gomes, W. R., and VanDemark, N. L. (1969). Oxygen tension in testes of normal and cryptorchid rats. *J Reprod Fert 19*, 559-561.

McCullen, S. D., Haslauer, C. M. and Loboa, E. G. (2010). Musculoskeletal mechanobiology: Interpretation by external force and engineered substratum. *J Biomech 43*, 119-127.

McKim, J. M. (2010). Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance. *Comb Chem High Throughput Screen 13*, 188-206.

Meistrich, M. L., Longtin, J., Brock, W. A., et al. (1981). Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. *Biol Reprod 25*, 1065-1077.

Midzak, A. S., Chen, H., Aon, M. A., et al. (2011). ATP synthesis, mitochondrial function, and steroid biosynthesis in rodent primary and tumor Leydig cells. *Biol Reprod 84*, 976-985.

Mieusset, R. and Bujan, L. (1995). Testicular heating and its possible contributions to male infertility: a review. *Intl J Androl 18*, 169-184.

Moritoki, Y., Kojima, Y., Mizuno, K., et al. (2012). Intratesticular pressure after testicular torsion as a predictor of subsequent spermatogenesis: a rat model. *Br J Urol 109*, 466-470.

Mruk, D. D. and Cheng, C. Y. (2004). Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr Rev 25*, 747-806.

Murphy, P. R. and Moger, W. H. (1982). Short-term primary culture of mouse interstitial cells: effects of culture conditions on androgen production. *Biol Reprod 27*, 38-47.

Mushtaq, M., Kulp, S., Chang, W., and Lin, Y. C. (1996). Gossypol inhibits human chorionic gonadotropin-stimulated testosterone production by cultured canine testicular interstitial cells. *Res Comm Molec Pathol Pharmacol 91*, 259-272.

Myers, R. B. and Abney, T. O. (1990). Testosterone and androstanediol production by regenerating Leydig cells in the ethylene dimethane sulphonate-treated rat. *Int J Androl 13*, 4-16.

NRC – National Research Council (2000). *Scientific Frontiers in Developmental Toxicology and Risk Assessment*. Washington, DC, USA: National Academies Press. http://www.nap.edu/catalog.php?record_id=9871

NRC (2007). *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*. Washington, DC, USA: National Academies Press. http://www.nap.edu/catalog.php?record_id=11970

Niemi, M. and Kormano, M. (1965). Cyclical changes and significance of lipids and acid phosphatase activity in the seminiferous tubules of the rat testis. *Anat Rec 151*,159-170.

Nolte, J., Michelmann, H. W., Wolf, M., et al. (2010). PSCDGs of mouse multipotent adult germline stem cells can enter and progress through meiosis to form haploid male germ cells in vitro. *Differentiation 80*, 184-194.

Nugraha, B., Hong, X., Mo, X., et al. (2011). Galactosylated cellulosic sponge for multi-well drug safety testing. *Biomaterials 32*, 6982-6994.

Olson, H., Betton, G., Robinson, D., et al. (2000). Concordance of the toxicity of pharmaceuticals in human and in animals. *Regul Toxicol Pharmacol 32*, 56-67.

Onoda, M., Suarez-Quian, C., Djakiew, D., and Dym, M. (1990). Characterization of Sertoli cells cultured in the bicameral chamber system: relationship between formation of permeability barriers and polarized secretion of transferrin. *Biol Reprod 43*, 672-683.

Orth, J. M., Jester, W. F., Li, L. H., and Laslett, A. L. (2000). Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. *Curr Top Dev Biol 50*, 103-124.

O'Shaughnessy, P. J. and Fowler, P. A. (2011). Endocrinology of the mammalian fetal testis. *Reproduction 141*, 37-46.

Parvinen, M. (1982). Regulation of the seminiferous epithelium. *Endocr Rev 3*, 404-417.

Parvinen, M., Toppari, J., and Lahdetie, J. (1993). Transillumination-phase contrast microscopic techniques for evaluation of male germ cell toxicity and mutagenicity. In R. E. Chapin

and J. Heindel (eds), *Methods in Reproductive Toxicology* (142-165). Orlando, FL, USA: Academic Press.

Patel, S. J., Milwid, J. M., King, K. R., et al. (2012). Gap junction inhibition prevents drug-induced liver toxicity and fulminant hepatic failure. *Nat Biotechnol 30*, 179-183.

Payne, A. H. and Hardy, M. P. (eds). (2007). *The Leydig Cell in Health and Disease*. Totowa, NJ, USA: Humana Press.

Piersma, A. H., Hernandez, L. G., Van Benthem, J., et al. (2011). Reproductive toxicants have a threshold of adversity. *Crit Rev Toxicol 41*, 545-554.

Quignot, N., Desmots, S., Barouki, R., and Lemazurier, E. (2012). A comparison of two human cell lines and two rat gonadal cell primary cultures as in vitro screening tools for aromatase modulation. *Toxicol In Vitro 26*, 107-118.

Rahman, N. A. and Huhtaniemi, I. T. (2004). Testicular cell lines. *Mol Cell Endocrinol 228*, 53-65.

Reader, S. C. J. and Foster, P. M. D. (1990). The in vitro effects of four isomers of dinitrotoluene on rat Sertoli and Sertoli-germ cell cocultures: germ cell detachment and lactate and pyruvate production. *Toxicol Appl Pharmacol 106*, 287-294.

Renato de Franca, L., Ghosh, S., Ye, S.-J., and Russell, L. D. (1993). Surface and surface-to-volume relationships of the Sertoli cell during the cycle of the seminiferous epithelium in the rat. *Biol Reprod 49*, 1215-1228.

Richburg, J. H., Redenbach, D. M., and Boekelheide, K. (1994). Seminiferous tubule fluid secretion is a Sertoli cell microtubule-dependent process inhibited by 2,5-hexanedione exposure. *Toxicol Appl Pharmacol 128*, 302-309.

Ritzén, E., Boitani, C., Parvinen, M., et al. (1982). Stage dependent secretion of ABP by rat seminiferous tubules. *Mol Cell Endocrinol 25*, 25-33.

Rosness, P. A., Sunde, A., and Eik-Nes, K. B. (1977). Production and effects of 7α-hydroxytestosterone on testosterone and dihydrotestosterone metabolism in rat testis. *Biochim Biphys Acta 488*, 55-68.

Rostami-Hodjegan, A. and Tucker, G. T. (2007). Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. *Nat Rev Drug Discov 6*, 140-148.

Russell, W. M. S. and Burch, R. L. (1959). *The Principles of Humane Experimental Technique*. London, UK: Methuen.

Russell, L. D. (1983). Normal testicular structure and methods of evaluation under experimental and disruptive conditions. In T. W. Clarkson, G. F. Nordberg, and P. R. Sager (eds). *Reproductive and Developmental Toxicity of Metals* (227-252). New York, USA. Plenum Pub Co.

Russell, L. D., Tallon-Doran, M., Weber, J. E., et al. (1983). Three-dimensional reconstruction of a rat stage V Sertoli cell: III. A study of specific cellular relationships. *Am J Anat 167*, 181-192.

Russell, L. D. and Peterson, R. N. (1985). Sertoli cell junctions: morphological and functional correlates. *Intl Rev Cyt 94*, 177-211.

Russell, L. D. and Steinberger, A. (1989). Sertoli cells in culture: Views from the perspective of an in vivoist and in vitroist. *Biol Reprod 41*, 571-577.

Russell, L. D. and Renato de Franca, L. (1995). Building a testis. *Tissue Cell 27*, 129-147.

Ryser, S., Glauser, D., Vigier, M., et al. (2011). Gene expression profiling of rat spermatogonia and Sertoli cells reveals signaling pathways from stem cells to niche and testicular cancer cells to surrounding stroma. *BMC Genomics 12*, 29-44.

Sato, T., Katagiri, K., Gohbara, A., et al. (2011). In vitro production of functional sperm in cultured neonatal mouse testes. *Nature 471*, 504-507.

Sasaki, J. C., Chapin, R. E., Hall, D. G., et al. (2011). Incidence and nature of testicular toxicity findings in pharmaceutical development. *Birth Defects Res B Dev Reprod Toxicol 92*, 511-525.

Scannell, J. W., Blanckley, A., Boldon, H., and Warrington, B. (2012). Diagnosing the decline in pharmaceutical R&D efficiency. *Nat Rev Drug Discov 11*, 191-200.

Schenk, B., Weimer, M., Bremer, S., et al. (2010). The RePro-Tect Feasibility Study, a novel comprehensive in vitro approach to detect reproductive toxicants. *Reprod Toxicol 30*, 200-218.

Schrag, S. D. and Dixon, R. L. (1985). Occupational exposures associated with male reproductive dysfunction. *Annu Rev Pharmacol Toxicol 25*, 567-592.

Schumacher, M., Schäfer, G., Holstein, A. F., and Hilz, H. (1978). Rapid isolation of mouse Leydig cells by centrifugation in Percoll density gradients with complete retention of morphological and biochemical integrity. *FEBS Lett 91*, 333-338.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol 13*, 29-83.

Setchell, B. P., Maddocks, S., and Brooks, D. E. (1994). Anatomy, vasculature, innervations, and fluids of the male reproductive tract. In E. Knobil and J. D. Neill (eds). *The Physiology of Reproduction* (1063-1175). 2nd edition. New York, USA: Raven Press.

Sharpe, R. M. (1989). Possible role of elongated spermatids in control of stage-dependent changes in the diameter of the lumen of the rat seminiferous tubule. *J Androl 10*, 304-310.

Si-Tayeb, K., Noto, F. K., Nagaoka, M., et al. (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology 51*, 297-305.

Siu, M. K. Y. and Cheng, C. Y. (2004). Extracellular matrix: recent advances on its role in junction dynamics in the seminiferous epithelium during spermatogenesis. *Biol Reprod 71*, 375-391.

Sivaraman, A., Leach, J. K., Townsend, S., et al. (2005). A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab 6*, 569-591.

Skinner, M. K. and Fritz, I. B. (1985). Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. *Proc Natl Acad Sci 82*, 114-118.

Skinner, M. K., Tung, P. S., and Fritz, I. B. (1985). Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. *J Cell Biol 100*, 1941-1947.

Sofikitis, N., Pappas, E., Kawatani, A., et al. (2005). Efforts to

create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment. *Hum Reprod Update 11*, 229-259.

Staub, C., Hue, D., Nicolle, J.-C., et al. (2000). The whole meiotic process can occur in vitro in untransformed rat spermatogenic cells. *Exp Cell Res 260*, 85-95.

Steinberger, E. and Ficher, M. (1968). Conversion of progesterone to testosterone by testicular tissue at different stages of maturation. *Steroids 11*, 351-368.

Steinberger, A. and Klinefelter, G. (1993). Sensitivity of Sertoli and Leydig cells to xenobiotics in in vitro models. *Reprod Toxciol 7*, 23-37.

Stukenborg, J.-B., Wistuba, J., Luetjens, C. M., et al. (2008). Co-culture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. *J Androl 29*, 312-329.

Su, L., Cheng, C. Y., and Mruk, D. D. (2010). Adjudin-mediated Sertoli-germ cell junction disassembly affects Sertoli cell barrier in vitro and in vivo. *Int J Biochem Cell Biol 42*, 1864-1875.

Tchounwou, P. B., Nwesome, C., Glass, K., et al. (2003). Environmental toxicology and health effects associated with dinitrotoluene exposure. *Rev Environ Health 18*, 203-229.

Tepe, S. J. and Zenick, H. (1984). The effects of carbon disulfide on the reproductive system of the male rat. *Toxicology 32*, 47-56.

Thyssen, B., Varma, S. K., and Bloch, E. (1985). Reproductive toxicity of 2,4-toluenediamine in the rat I. Effect on male fertility. *J Toxicol Environ Health 16*, 753-762.

TSCA – Toxic Substances Control Act, 15 U.S.C. §2601 et seq. (1976). *Trends Biochem Sci 29*, 364-370.

Toh, Y. C., Zhang, C., Zhang, J., et al. (2007). A novel 3D mammalian cell perfusion-culture system in microfluidic channels. *Lab Chip 7*, 302-309.

Toh, Y. C., Blagovic, K., Yu, H., and Voldman, J. (2011). Spatially organized in vitro models instruct asymmetric stem cell differentiation. *Integr Biol 3*, 1179-1187.

Tostoes, R. M., Leite, S. B., Serra, M., et al. (2012). Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. *Hepatology 55*, 1227-1236.

Ulbrich, B. and Palmer, A. K. (1995). Detection of effects on male reproduction – a literature survey. *J Am College Tox 14*, 293-327.

van der Burg, B., Kroese, E. D., and Piersma, A. H. (2011). Towards a pragmatic alternative testing strategy for the detection of reproductive toxicants. *Reprod Toxicol 31*, 558–561.

van der Jagt, K., Munn, S., Tørsløv, J., and de Bruijn, J. (2004). Alternative approaches can reduce the use of animals under REACH. http://publications.jrc.ec.europa.eu/repository/

Verhoeven, G. and Cailleau, J. (1985). A factor in spent media from Sertoli cell-enriched cultures that stimulates steroidogenesis in Leydig cells. *Molec Cell Endocrinol 40*, 57-68.

Verhoeven, G. and Cailleau, J. (1988). Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells. *Endocrinol 123*, 2100-2110.

Verhoeven, G., Cailleau, J., and DeClercq, E. (1988). Stimulatory effects of antiviral adenosine analogs on steroidogenesis in Leydig cells. *J Steroid Biochem 31*, 267-274.

Verhoeven, G., Cailleau, J., and Morris, I.D. (1989). Inhibitor effects of alkane sulphonates on the function of immature rat Leydig, Sertoli and peritubular cells cultured in vitro. *J Molec Endocrinol 2*, 145-155.

Veulemans, H., Steeno, O., Masschelein, R., and Groeseneken, D. (1993). Exposure to ethylene glycol ethers and spermatogenic disorders in man: a case-control study. *Br J Ind Med 50*, 71-78.

Von Schonfeldt, V., Wistuba, J., and Schlatt, S. (2004). Notch-1, c-kit and GFRα-1 are developmentally regulated markers for premeiotic germ cells. *Cytogenet Genome Res 105*, 235-239.

Vreeburg, J. T. M., Ooms, M. P., Rommerts, F. F. G., and Teerds, K. J. (1988). Functional properties of developing rat Leydig cells after treatment with ethylene dimethanesulphonate (EDS). *J Reprod Fert 84*, 63-69.

Walther, N., Jansen, M., Ergun, S., et al. (1996). Sertoli cell lines established from H-2Kb-tsA58 transgenic mice differentially regulate the expression of cell-specific genes. *Exp Cell Res 225*, 411-421.

Warren, D. W., Wisner, J. R., and Ahmad, N. (1984). Effects of 1,2-Dibromo-3-Chloropropane on male reproductive function in the rat. *Biol Reprod 31*, 454-463.

Weber, J. E., Russell, L. D., Wong, V., and Peterson, R. N. (1983). Three-dimensional reconstruction of a rat stage V Sertoli cell: II. Morphometry of Sertoli-Sertoli and Sertoli-germ-cell relationships. *Am J Anat 167*, 163-179.

Wegner, S., Hong, S., Yu, X., and Faustman, E. M. (2013). Preparation of rodent testis co-cultures. *Curr Protoc Toxicol,* 16.10.1-16.10.7.

Wen, F., Chang, S., Toh, Y. C., et al. (2008). Development of dual-compartment perfusion bioreactor for serial co-culture of hepatocytes and stellate cells in poly (lactic-co glycolic acid)-collagen scaffolds. *J Biomed Mat Res B Appl Biomat 87*, 154-162.

Whorton, D., Krauss, R. M., Marshall, S., and Milby, T. H. (1977). Infertility in male pesticide workers. *Lancet 2*, 1259-1261.

Williams, J. and Foster, P. M. D. (1988). The production of lactate and pyruvate as sensitive indices of altered rat sertoli cell function in vitro following the addition of various testicular toxicants. *Toxicol Appl Pharmacol 94*, 160-170.

Wing, T. Y. and Christensen, A. K. (1982). Morphometric studies on rat seminiferous tubules. *Am J Anat 165*, 13-25.

Wright, W. W., Musto, N. A., Mather, J. P., and Bardin, C. W. (1981). Sertoli cells secrete both testis-specific and serum proteins. *Proc Natl Acad Sci 78*, 7565-7569.

Wright, W. W., Parvinen, M., Musto, N. A., et al. (1983). Identification of stage-specific proteins synthesized by rat seminiferous tubules. *Biol Reprod 29*, 257-270.

Wong, C. C. S., Chung, S. S. W., Grima, J., et al. (2000). Changes in the expression of junctional and nonjunctional complex component genes when inter-Sertoli tight junctions are formed in vitro. *J Androl 21*, 227-237.

Yao, P.-L., Lin, Y.-C., and Richburgh, J. H. (2010). Mono-(2-ethylhexyl) phthalate-induced disruption of junctional complexes in the seminiferous epithelium of the rodent testis is mediated by MMP2. *Biol Reprod 82*, 516-527.

Yang, J. M., Arnush, M., Chen, Q. Y., et al. (2003). Cadmium-induced damage to primary cultures of rat Leydig cells. *Reprod Toxicol 17*, 553-560.

Yee, J. B. and Hutson, J. (1985). Effects of testicular macrophage-conditioned medium on Leydig cells in culture. *Endocrinol 116*, 2682-2684.

Yu, X., Sidhu, J. S., Hong, S., and Faustman, E. M. (2005). Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat Sertoli cell/gonocyte co-cultures: an improved in vitro model for assessment of male reproductive toxicity. *Toxicol Sci 84*, 378-393.

Yu, X., Hong, S. W., Moreira, E. G., and Faustman, E. M. (2009). Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. *Toxicol Appl Pharmacol 239*, 325-336.

Yu, H. and Rahim, N. A. A. (2013). *Introduction to Cellular and Tissue Engineering* (CTE). Boca Raton, FL, USA: CRC Press Taylor & Francis.

Yue, Z., Wen, F., Gao, S., et al. (2010). Preparation of three-dimensional interconnected macroporous cellulosic hydrogels for soft tissue engineering. *Biomaterials 31*, 8141-8152.

Zhang, S., Xia, L., Kang, C. H., et al. (2008). Microfabricated silicon nitride membranes for hepatocyte sandwich culture. *Biomaterials 29*, 3993-4002.

Zhang, C., Chia, S. M., Ong, S. M., et al. (2009a). The controlled presentation of TGF-beta1 to hepatocytes in a 3D-microfluidic cell culture system. *Biomaterials 30*, 3847-3853.

Zhang, C., Zhao, Z., Abdul Rahim, N. A., et al. (2009b). Towards a human-on-chip: Culturing multiple cell types on a chip with compartmentalized microenvironments. *Lab Chip 9*, 3185-3192.

Zhang, S., Tong, W., Zheng, B., et al. (2011). A robust high-throughput sandwich cell-based drug screening platform. *Biomaterials 32*, 1229-1241.

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