Appendix 1: Single and co-culture cell cultures

Assay type & source	Findings
Single cell	
Leydig cells	For Leydig cell cultures, crude collagenase digestion of the testes yields 5-15% Leydig cells, while enzymatic digestion yields 55-85% Leydig cells, and centrifugal elutriation yields samples that are >90% Leydig cells. The most common species utilized for obtaining Leydig cells are the mouse (Schumacher et al., 1978), rat (Verhoeven et al., 1988; Ku et al., 1993; Quignot et al., 2012), and pig (Brun et al., 1991). The sexual maturity of the donor animals influences the parameters that can be evaluated. A variation of this approach involves the use of cultured cell lines (e.g., immortalized mouse tumor Leydig cells (MA-10; Kotula-Balak et al., 2011)). Leydig cell function, such as testosterone and progesterone production and cAMP biosynthesis, can be evaluated in response to exposure to chemicals of interest (Steinberger and Klinefelter, 1993). Additional references: Verhoeven et al. (1989); Rahman and Huhtaniemi (2004); Chapin and Phelps (1990)
Interstitial cells	Similarly, interstitial cells can be harvested from testes that have been enzymatically digested. The most common species utilized for obtaining interstitial cells are the mouse (Murphy and Moger, 1982), rat (Verhoeven et al., 1988), and dog (Mushtaq et al., 1996), although other species such as bull and boar (Mushtaq et al., 1996) also have been investigated. Culture of interstitial cells, which include Leydig cells, allows one to evaluate the potential effects of compounds on testosterone secretion.
Sertoli cells	The most commonly used single cell cultures utilize Sertoli cells. Unless they are specifically removed, all Sertoli cell cultures contain some germ cells, depending on the age of the donor animals, since older animals have more germ cells than younger (Chapin and Phelps, 1990). The use of a two-compartment culture system simulates physiologic conditions in which the formation of a confluent, highly polarized monolayer with intercellular tight junctions that mimic the blood-testis barrier *in vivo* (Steinberger and Klinefelter, 1993). Sertoli cells secrete lactate and pyruvate, which are essential for maintenance and growth of developing germ cells *in vivo*. Accordingly, lactate and pyruvate levels are routinely measured in spent Sertoli cell media, as well as other biochemical responses (Skinner and Fritz, 1985; Skinner et al., 1985; Chapin et al., 1991). Cultured Sertoli cells also display several other expected responses: FSH-induced production of c-AMP, FSH-induced aromatase activity, and androgen binding protein production (Verhoeven and Cailleau, 1988; Allenby et al., 1991a,b). A rat Sertoli cell line (SerW3) has been established that displays the morphology, including tight junctions, and some functional features of Sertoli cells *in vivo* (Fiorini et al., 2004). Additional references: Lamb and Chapin, 1993; Verhoeven et al., 1988
Germ cells	Examples of germ cells used for culture include pachytene spermatocytes (Meistrich et al., 1981; Holmes et al., 1983; Jutte et al., 1985), round spermatids (Meistrich et al., 1981; Grootegoed et al., 1982), enriched mixed germ cells (Ku and Chapin, 1994), and germline stem cells (SCC; Dann et al., 2008; Marcon et al., 2010; Heim et al., 2011). Cultures of germ cells that are highly enriched for one cell type are appropriate when the cell type most sensitive to a toxicant has already been identified by *in vivo* studies (Chapin and Phelps, 1990; Kotaja et al., 2004). This allows investigation of biochemical or molecular pathways that might be involved.
Peritubular cells	Peritubular cell cultures can be used to evaluate the effects of compounds on the production of paracrine factors, P Mod-S (proteins secreted by Peritubular cells that Modulate Sertoli cell function) involved in testicular function, such as those modulating the production of androgen binding protein or transferring that are produced by Sertoli cells (Verhoeven and Cailleau, 1988; Verhoeven et al., 1988). They also have been used to investigate other biochemical responses (Skinner and

Assay type & source	Findings
	Fritz, 1985; Skinner et al., 1985).
Co-cultures Sertoli-germ cells	One of the earliest examples is co-culture of Sertoli and germ cells (Gray, 1986). In this model, the Sertoli cells attach to the substrate and form a confluent monolayer, with the germ cells attaching to the Sertoli monolayer. Effects on Sertoli cells or detachment of the germ cells from the Sertoli monolayer serve as indicators of potential testicular toxicity (Cave and Foster, 1990; Chapin and Phelps, 1990; Allenby et al., 1991a,b; Lamb and Chapin, 1993). In addition, this model can be used to evaluate the effects of chemicals on such biochemical changes as FSH-stimulated intracellular cAMP accumulation in the Sertoli cell (Ku et al., 1993) and cell viability (Adhikari et al., 2000). Recent studies by Yu and colleagues (Yu et al., 2005, 2009) have indicated that extracellular matrix plays a critical role in germline stem cell (gonocyte) self-renewal and progeny production. Accordingly, they have developed a Sertoli cell-gonocyte culture model with an extracellular matrix (Matrigel) applied as an overlay instead of a substratum (Yu et al., 2005, 2009). This system led to enhanced attachment of the Sertoli cells and facilitated establishment of intercellular communication and cytoskeletal structure, thereby greatly increasing cell viability. These investigators also have used this new system to study the toxicogenomic effects of testicular toxicants. Also see Ku and Chapin, 1994.
Sertoli-peritubular cells	Sertoli-peritubular co-cultures have been used to study altered paracrine secretion and other biochemical responses compared with monocultures of Sertoli or peritubular cells (Skinner and Fritz, 1985; Skinner et al., 1985). The role of Sertoli and peritubular interactions in the control of Sertoli cell function by androgens was studied by Verhoeven and Cailleau (1988). Parameters of androgen action were decreased FSH-inducible aromatase activity and increased secretion of androgen-binding protein.
Leydig-Sertoli cells	Single-cell models are useful in identifying targets and ultimate toxicants. However, co-cultures allow the researchers to test whether a metabolite or other factor produced in one cell can affect an adjacent and more visibly responsive cell type (Chapin et al., 1990; Lamb and Chapin, 1993). Using this system allowed investigators to determine that spent media from Sertoli cell cultures contains a paracrine factor that stimulates early steroidogenesis in Leydig cells but also interferes with the conversion of C_{21} precursors into androgens (Verhoeven and Cailleau 1985).
Leydig-macrophage	Similarly, the effects of testicular macrophage-conditioned medium on Leydig cells can be studied in a co-culture system, whereby testosterone production is stimulated by the conditioned medium when added to Leydig cells *in vitro* (Yee and Hutson, 1985).
Seminiferous tubules	Cultured seminiferous tubules can be used to investigate the effects of potential testicular toxicants on inhibin as an indicator of early toxicant action on spermatogenesis (Allenby et al., 1991a,b). Inhibin is a Sertoli cell hormone whose blood levels decrease following disruption of spermatogenesis. Therefore, it has been proposed as a biomarker of testicular toxicity. In addition, one can detect the structural changes in spermatocytes that are associated with cell degeneration (Lamb and Chapin, 1993). Ku and Chapin (1994) reported that intact seminiferous tubule structure *in vitro* was required for full expression of spermatocyte toxicity to be similar between 2-methoxyethanol (ME) and its active metabolite, 2-methoxyacetic acid (MAA). Specifically, the morphologic degeneration observed in germ cells exposed to ME was not present after MAA exposure unless intact seminiferous tubules were present in the culture system.
Fetal testis	Chauvigne et al. (2011) have used an organotypic culture system for fetal rat gonads obtained on Gestation Day 14.5 to study direct endocrine disruptor-induced testicular dysfunction. A mechanistic investigation was conducted of phthalate-induced alteration of testicular function. However, the authors report that the culture procedure and analysis are tedious and time-consuming.

Assay type & source	Findings
[3-D models]	See 3D section
Germ cell culture	Germ cells Sofikitis et al., 2005

Appendix 2: 2 Chamber Culture System

Compound	Objective	Culture system	Concentration(s) tested	Observations	Reference
Cadmium Chloride ($CdCl_2$)	Use 2-chamber Sertoli cell culture system to determine if *in vivo* deleterious effects of $CdCl_2$ on Sertoli cell tight junction could be observed *in vitro*.	Sertoli cells from 18-day-old Sprague Dawley rats as single cells or small cell aggregated on Matrigel-covered filters in two-compartment culture system for up to 13 days.	0.75 - 24 µM for 4 or 18 h on Day 1 or 5 of culture.	Concentration dependent decrease in TER with the effect being somewhat great if added after 5 days in culture. Decreases in TER are reflective of disruption of Sertoli tight junctions, as demonstrated *in vivo*.	Janecki et al., 1992
$CdCl_2$	Using the known Sertoli cell toxin, $CdCl_2$, determine if the Sertoli cell 2-chamber culture system could be used to more closely assess effects on BTB.	Sertoli cells from 20-day-old Sprague Dawley rats at low and high density cultures on Matrigel-covered filters in two-compartment culture system for up to 8 days.	0.1 - 10 µM for 8 h on days 1 and 5 of culture.	Effects produced by $CdCl_2$ on Sertoli cell tight junctions and proteins associated with tight junctions were as expected in that the effects were concentration/time dependent and reversible in the presence of testosterone. These effects thereby justify that this culture system can be used to assess effects of compound on BTB *in vitro*.	Chung and Cheng, 2001
Hexane Chromium	Use 2-chamber seminiferous cell culture system to examine deleterious effects of chromium on germ cell meiosis.	Sertoli and germ cells from 23-day-old Wistar rats were cultured on Matrigel-coated filters in the inner well of 2-chamber culture system for up to 18 days and compared to *in vivo* effects observed in adults	1, 10, or 100 µg/l added on day 2 of culture and maintained for up to 18 days.	Chromium added to the outer well was able to traverse to cells in the inner well resulting in concentration and time related alterations in chromosomes. These results, in general, reflect abnormalities produced to germ cells of rats exposed to chromium *in vivo*.	Geoffroy-Siraudin et al., 2010

Compound	Objective	Culture system	Concentration(s) tested	Observations	Reference
Adjudin	Utilize *in vivo* and *in vitro* treatment with Adjudin to assess effects on blood-testis-barrier.	Sertoli cells from 20-day-old Sprague Dawley rats were cultures at high density on Matrigel-covered filters in two-compartment culture system and compared to results from treated adult rats.	0.1, 0.5, and 1 µg/ml *in vitro* for up to 7 days compared to single oral dose of 50 mg/kg.	In general, exposure of Sertoli cell *in vitro* produced effects similar to those observed *in vivo* and comprising a strengthening of the tight junctions between adjacent Sertoli cells and increase in associated proteins.	Su et al., 2010
Bisphenol A	Use *in vitro* Sertoli cell 2-chamber culture system to assess reversible effects of bisphenol of blood-testis-barrier and compare to *in vivo* effects.	Sertoli cells from 20-day-old Sprague Dawley rats were cultured in 2-chamber culture system and results compared to those from *in vivo* treated adult Wistar rats.	0.01 to 800 µM *in vitro* and 0.02 to 50 mg/kg *in vivo*.	Disruption of blood-testis-barrier was similar when immature rats (or Sertoli cells from immature rats) were exposed to bisphenol A. Adult testes do not show effects on blood-testis-barrier.	Li et al., 2009

TER – Transepithelial electrical resistance