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Xenotransplantation Models to Study the Effects of Toxicants on Human Fetal Tissues¹

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Abstract

Many diseases that manifest throughout the lifetime are influenced by factors affecting fetal development. Fetal exposure to xenobiotics, in particular, may influence the development of adult diseases. Established animal models provide systems for characterizing both developmental biology and developmental toxicology. However, animal model systems do not allow researchers to assess the mechanistic effects of toxicants on developing human tissue. Human fetal tissue xenotransplantation models have recently been implemented to provide human-relevant mechanistic data on the many tissue-level functions that may be affected by fetal exposure to toxicants. This review describes the development of human fetal tissue xenotransplant models for testis, prostate, lung, liver, and adipose tissue, aimed at studying the effects of xenobiotics on tissue development, including implications for testicular dysgenesis, prostate disease, lung disease, and metabolic syndrome. The mechanistic data obtained from these models can complement data from epidemiology, traditional animal models, and *in vitro* studies to quantify the risks of toxicant exposures during human development.

Keywords

development; xenobiotics; toxicity; xenotransplants

Introduction

Unique windows of sensitivity occur in early life, particularly *in utero*, during which toxicants can disrupt critical developmental processes. While some *in utero* exposures to toxicants can disrupt tissue growth and differentiation, resulting in overt teratogenicity, the

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effects of some *in utero* toxicant exposures may have a more subtle influence on health status at birth, as well as later in life. For instance, Dr. David Barker and colleagues extensively documented connections between reduced maternal nutrition and impaired fetal and infant development. This impairment leads to an increased risk of developing cardiovascular disease and diabetes later in life (Barker and Osmond, 1986; Barker et al., 1989; Hales et al., 1991; Barker et al., 1993; Barker, 1995; Fall et al., 1995a; Fall et al., 1995b; Fall et al., 1995c; Law et al., 1995; Martyn et al., 1995; Vijayakumar et al., 1995; Yajnik et al., 1995; Barker, 1997; Barker and Martyn, 1997). Several reviews have addressed diseases hypothesized to originate in altered fetal development, including metabolic syndrome (Gluckman and Hanson, 2004b) and the disorders of male reproduction known as Testicular Dysgenesis Syndrome (Skakkebaek et al., 2001). Fetal exposure to stressors such as poor nutrition and environmental toxicants may promote later life diseases through multiple mechanisms, including alterations in the epigenome (Gluckman and Hanson, 2004a; Dolinoy et al., 2007; Jirtle and Skinner, 2007; Szyf, 2007; Perera and Herbstman, 2011; Cortessis et al., 2012).

Numerous animal models are used to study developmental biology, chief among them the fruit fly (*Drosophila melanogaster*), the nematode (*Caenorhabditis elegans*), the chicken (*Gallus gallus*), the mouse (*Mus musculus*), the frog (*Xenopus laevis*), and the zebrafish (*Danio rerio*) (Jenner and Wills, 2007). These model organisms have all been successfully utilized to study aspects of toxicant response during development (Lewandowski et al., 2003; Boyd et al., 2009; Ruden et al., 2009; Jiang et al., 2012; Bailey et al., 2013; Kim et al., 2013). However, biological differences between animals and humans can pose a problem in extrapolating the toxicological response mechanisms identified in these model organisms to humans. Although animal models, especially rodents, have provided accurate modeling for many biological processes, both normal and disease, it is worth noting that one in five human genes has no orthologue in the mouse model system (Church et al., 2009). The response to toxicant exposure has been shown to vary across species, in part because it can be dramatically influenced by polymorphisms or expression differences in receptors, target genes, transporters, or metabolic enzymes [e.g. (Kudo and Kawashima, 2003; Gonzalez and Shah, 2008; Graham and Lake, 2008; Sharma and McNeill, 2009)]. Species differences in response to developmental toxicants range from quantitative to more significant qualitative differences. For example, Lewandowski et al. (2003) reported quantitative species differences in the degree of neurotoxic effect of methylmercury exposure during development, with humans being more sensitive than the mouse, which was more sensitive than the rat. Conversely, there is a qualitative species difference in response to thalidomide. Thalidomide is a severe teratogen in humans, but not in rodents; this particular species difference may be attributable to metabolic differences (Bauer et al., 1998; Vargesson, 2009). In pharmaceutical toxicity testing, one analysis indicated that 70% of human toxicities were predicted by preclinical toxicities in animal studies, indicating a high rate of screening success but a significant potential for species differences (Olson et al., 2000). Because of the potential for such qualitative species differences, developmental hazard identification in regulatory toxicology relies on a process of testing one rodent and one non-rodent species (OECD, 2001). However, in many cases alternative model systems could

provide a greater degree of relevance to human biology, particularly when the goal of a study is to gain a deep mechanistic understanding of the human response to a toxicant.

In a xenotransplant model, human cells, tissues, or organs can be implanted into an immunodeficient animal host, which provides the necessary supportive environment for the human tissue to function and grow normally. Researchers have transplanted a number of rodent and human tissues, organs, and cell types into rodent hosts. These tissues include testis (Kuopio et al., 1989; Arregui and Dobrinski, 2014), ovary (Bartholomeusz et al., 2012; Scott et al., 2013), breast (Clarke, 2009; Mollard et al., 2011), thymus, liver, lymph node (McCune et al., 1988; Denton and Garcia, 2009), prostate (Sugimura et al., 1988; Yonemura et al., 1995; Wang et al., 2005), and lung (Pavlovic et al., 2008). Xenotransplantation models have been used to test cancer therapies (Morton and Houghton, 2007; Clarke, 2009; Mollard et al., 2011; Luconi and Mannelli, 2012; Ruggeri et al., 2014) and to study a variety of diseases such as diabetes, asthma, HIV, and other infectious diseases, described in reviews by Brehm et al. (2010) and Shultz et al. (2007). Xenotransplant models of human fetal tissues including lung (Cobb, 1975; Peault et al., 1994; Berin et al., 2001; Pavlovic et al., 2008; Valbuena et al., 2014), trachea (Keswani et al., 2011; Keswani et al., 2012), pancreas (Capito et al., 2013), intestine (Golan et al., 2011), testis (Mitchell et al., 2010), and ovary (Poulain et al., 2014) have been developed to study aspects of normal biology and disease.

In this review, we describe our recent development of xenotransplant models with the goal of studying the effects of xenobiotics in human fetal testis (Heger et al., 2012; Spade et al., 2014), prostate (Saffarini et al., 2013), lung (De Paepe et al., 2012a; De Paepe et al., 2012b), liver, and adipose tissue (Garcia et al., 2014). We do not aim to conduct an exhaustive review of the extensive literature on xenotransplantation. Rather, we will describe the development and utility of xenotransplantation models to contribute to human toxicology studies, with respect to the effects of toxicant exposures on the development of fetal tissues. The majority of our xenotransplant studies have shown that the tissue is supported by the rodent host environment, maintains the ability to respond to endocrine factors, and responds to toxicant exposures. However, model development remains a significant challenge and must be tailored to the particular tissue and research question. Given appropriate development and validation, these models have shown promise for the study of mechanistic responses to toxicants at the tissue level. This mechanistic data can contribute to the body of toxicology literature from epidemiology and animal studies and guide endpoint selection and risk assessment. The factors that influence model optimization and research success will be discussed, along with research results to date, challenges, and future directions.

Goals of Human Fetal Tissue Xenotransplantation Studies in Toxicology

Develop and characterize human fetal tissue xenotransplant models

The goal of developing and fully characterizing human fetal tissue xenotransplant models is underway and will be described for several tissues in the following sections. In addition to the human fetal testis (Heger et al., 2012; Spade et al., 2014), prostate (Saffarini et al., 2013), lung (De Paepe et al., 2012a; De Paepe et al., 2012b), liver, and adipose tissue (Garcia et al., 2014) xenotransplant models we have developed specifically for toxicological

studies, there are other xenotransplant models for human fetal tissues including lung (Cobb, 1975; Peault et al., 1994; Berin et al., 2001; Pavlovic et al., 2008; Valbuena et al., 2014), trachea (Keswani et al., 2011; Keswani et al., 2012; Valbuena et al., 2014), pancreas (Capito et al., 2013), intestine (Golan et al., 2011), testis (Mitchell et al., 2010), and ovary (Poulain et al., 2014), which have been used to address a variety of scientific questions. The human fetal tissue xenotransplantation approach can be applied to numerous tissues, and there are likely to be toxicological applications for which these models and others can be applied.

Investigate human fetal tissue response to environmental stressors in xenotransplant models

In a xenotransplant model, the toxicant of interest can be introduced into a system containing functional human fetal tissue. As discussed above, the response to toxicants can differ among species, thus while animal models in developmental toxicology provide a great deal of data on the toxicity of chemicals in intact systems, including data on species differences in metabolism, they may not accurately predict the mechanistic responses of human fetal tissues to toxicants. Therefore, it is a goal of xenotransplant studies in toxicology to describe the molecular and cellular biology and biochemistry of human fetal tissues at various stages of development in order to assess the potential perturbations of those mechanisms by xenobiotics. Success in this effort would provide toxicity data complementary to that obtained from animal models, *in vitro* systems, and epidemiological studies. Data obtained from human fetal xenotransplantation may also guide endpoint selection in animal studies and contribute to risk assessment.

Investigate the effects of fetal toxicant exposure on later life diseases

Developing fetal tissue epigenomes are susceptible to significant and permanent change in response to environmental perturbations (Dolinoy et al., 2007; Jirtle and Skinner, 2007; Szyf, 2007; Perera and Herbstman, 2011). Despite evidence that exposure to toxicants or other adverse conditions during fetal life is associated with later life disease in humans, many of the mechanisms connecting exposures and outcomes remain unclear. A unique exception is the connection between metabolic effects of the Dutch Hunger Winter and the hypomethylation of the *IGF2* gene in those exposed to famine during fetal development (Heijmans et al., 2008). Xenotransplantation models provide an opportunity to study mechanistic aspects of the genomic and epigenomic response to toxicants in human tissues in an experimental setting. An ultimate goal for human xenotransplant models in toxicology is to perform dose-response studies to quantify the effects of toxicants on various fetal tissue epigenomes to provide insight into critical loci where the epigenomic controls of gene expression might be permanently altered.

Considerations for Development of Xenotransplantation Models

Successful xenotransplant model development is contingent upon identifying the research goals of the study, the needs of the tissue being transplanted, and the limitations of the host environment. Researchers must select an appropriate host species and strain, identify the appropriate site of transplantation, and optimize the model for the particular parameters of the transplant tissue, including tissue age, latency, and endocrine activity. Researchers must

also address ethical concerns involving responsible treatment of research animals and human tissue use. The xenotransplant study procedure illustrated in Figure 1 requires careful planning and optimization. This review will draw on our recent characterization of human fetal testis, prostate, lung, and adipose tissue models (Figure 2) as examples of the requirements for model development.

Host Selection

Immunocompromised rodents are used as hosts for xenotransplantation studies to avoid graft rejection. Common immunodeficient mice used as hosts for xenotransplantation are discussed in the review by Shultz et al. (2007). The degree of host immunodeficiency required for the study may vary by tissue. For instance, athymic nude mice and RNU rats are T-cell deficient, which is sufficient for some human fetal tissues, including testis (Heger et al., 2012; Spade et al., 2014) and prostate (Saffarini et al., 2013). More severely immunocompromised hosts such as SCID Beige mice may be optimal for other tissues, as is the case for transplantation of human fetal lung (De Paepe et al., 2012a). The choice between using a rat or a mouse as a host may depend on issues of scale, such as the available space at the xenotransplantation site and the host blood volume for metabolite measurement. The choice of a host may also be influenced by the available data on differential rat and mouse nutrient use and drug metabolism. Comparative species data on drug metabolism may be available in the literature, but can also be addressed through xenotransplant experimental design. For instance, to address concerns about metabolism of di-*n*-butyl phthalate to the active metabolite mono-*n*-butyl phthalate, Mitchell et al. (2012) performed xenotransplant experiments with both the parent compound and the metabolite.

Xenotransplantation Procedure

The site of transplantation is important for successful grafting and subsequent recovery of the transplanted tissue. In particular, xenotransplanted tissues require adequate access to the vascular supply of the host. Human and animal tissues have been successfully xenotransplanted into rodent hosts using orthotopic, intraperitoneal, subcutaneous, and renal subcapsular transplant sites. This review will primarily discuss the latter two sites. However, orthotopic xenotransplants have been successfully developed using normal and cancerous tissues and cells of the prostate (Wang et al., 2005), gallbladder (Egberts et al., 2007), bone (Vormoor et al., 2014), pancreas (Walters et al., 2013), ovary (Bartholomeusz et al., 2012), and liver (Strom et al., 2010; Sia et al., 2013). The latter has been proposed as a toxicological model (Strom et al., 2010). Intraperitoneal transplantation has been reported with, for example, pancreatic islets (Lanza et al., 1991) and cultured ovarian clear cell carcinoma cells (Bartholomeusz et al., 2012).

As an example of xenotransplantation methodology, Morton and Houghton (2007) describe a method for subcutaneous grafting of human tumor tissue in mice. Subcutaneous transplantation of human tissues has been studied extensively, including transplantation of normal human testis tissue. In a study of human adult testis xenotransplants in mouse hosts, 76% of xenotransplants were successfully retrieved from the host at the conclusion of the study, some of which maintained some potential for spermatogenesis (Schlatt et al., 2006). Schlatt et al. (2010) also showed that subcutaneous transplants of postnatal day 7 rat testis

form new transplant-derived vascular connections with the host. Recent toxicology studies utilizing a subcutaneous human fetal testis xenotransplantation model reported normal fetal testicular morphology and a 70–79% xenotransplant retrieval rate (Mitchell et al., 2010; Mitchell et al., 2012; Mitchell et al., 2013). Subcutaneous transplantation offers the advantage of being an easier and less time-consuming surgical procedure than renal subcapsular transplantation. However, the renal subcapsular location may be more advantageous for other reasons.

Renal subcapsular xenotransplants in comparison to subcutaneous xenotransplants have superior access to the host vasculature (Wang et al., 2005). In our studies, human fetal testis, lung, adipose tissue, and prostate xenotransplants maintain donor tissue-derived vascularization, as evidenced by immunohistochemical localization of the human endothelial marker CD31 (De Paepe et al., 2012a; Heger et al., 2012; Saffarini et al., 2013; Garcia et al., 2014) (Figure 2). In fetal lung and adipose tissue, we have further demonstrated that human CD31-positive blood vessels extend from the xenotransplant into the renal cortex of the host (De Paepe et al., 2012a; Garcia et al., 2014) (Figure 2). In the human fetal lung xenotransplant model, the renal subcapsular site supports more developmentally appropriate fetal lung architecture than the subcutaneous site, as evidenced by a significantly higher density of vascular tissue, greater surface area for gas exchange at the lung parenchyma, and greater expression of mRNAs involved in vascular tissue growth and development (De Paepe et al., 2012a). Renal subcapsular prostate xenotransplants survive for at least 200 days in the host (Saffarini et al., 2013).

Tissue Sample Characteristics

Success in xenotransplantation experiments is influenced by characteristics of the tissue sample including latency, developmental age, and endocrine factors. Viability of the transplant tissue is affected by latency from tissue acquisition until transplantation into the host. In the case of fetal tissue xenotransplants this latency period may include the time from fetal death until delivery, which is difficult to estimate. However, the time from delivery until xenotransplantation can be controlled and for human fetal testis has been limited to 36 hours (Heger et al., 2012; Spade et al., 2014), given that rat testis deteriorates significantly at longer time points (Bryant and Boekelheide, 2007). In our fetal tissue xenotransplant experiments, samples derived from macerated fetuses were excluded, as maceration indicates prolonged in utero retention of the fetus following demise. Lung transplants were generally performed within 36 hours after delivery. However, in one exceptional case, we achieved a successful transplant even 41 hours after delivery of the fetus (De Paepe et al., 2012a; De Paepe et al., 2012b). Cryopreservation of testis tissues has been attempted as a strategy to preserve testis for future xenotransplantation and production of spermatozoa *ex situ* with some success (Ehmcke and Schlatt, 2008). However, there is currently no data to indicate whether this approach would adversely affect germ cell survival in later xenotransplantation experiments.

The developmental age of fetal tissue samples can have significant consequences for both morphology and response to toxicants in xenotransplantation studies. For instance, human fetal lung xenotransplants differ morphologically with age and exhibit a greater potential to

produce progenitor lung epithelial cells at 13–17 weeks gestation than at 19–22 weeks gestation (De Paepe et al., 2012b). In the testis, developmental age affects endocrine processes (Scott et al., 2009) and cell growth, as well as the status of the germ line epigenome (Reik et al., 2001). These differences may affect response to toxicants. Scott et al. (2009) have proposed that the masculinization programming window during which human fetal males are most susceptible to anti-androgenic exposures occurs during gestational weeks eight to 15, but that exposures after this time likely still have adverse effects. As previously discussed, the fetal epigenome is particularly sensitive to toxicant exposures, and epigenomic changes during this period can affect later life health and disease (Perera and Herbstman, 2011). To fully understand the mechanisms by which fetal exposures can affect human health, these changing biological processes must be accounted for both in design and interpretation of toxicology studies.

As in all studies of developing tissue, whether in xenotransplants, *in vitro*, or in animal models, developmental age must be carefully considered in the design of the experiment to obtain meaningful and relevant results. In this regard, the human tissue xenotransplantation approach offers an advantage in design and analysis, if implemented carefully. A single piece of donor tissue is typically large enough to be dissected into multiple pieces, which can be grafted into multiple hosts. Those hosts then bear tissue from the same biological individual and can be treated as paired samples in the study. Those paired samples can be divided among treatment groups to control for some of the genetic variability among tissue samples. This design also gives an increase in statistical power, as a paired *t*-test or repeated-measures ANOVA is more powerful than the corresponding unpaired design. This design allows for sample ages to be distributed across treatment groups. However, researchers should assess control samples for age-based differences in morphology or response and should also consider any knowledge of tissue ontogeny in the literature that may affect study design.

If the organ or tissue to be studied in a xenotransplantation model is a source or target of circulating hormones, then the host endocrine system should be carefully considered in model development. For example, prostate xenografts in an intact adult rat host environment demonstrated accelerated maturation within 200 days, presenting as tissue that phenotypically resembled the adult prostate, likely owing to the hormonal status of the host (Saffarini et al., 2013). A castrate host, stimulated with human chorionic gonadotropin (hCG), has been used to promote testosterone production in several testis xenotransplant studies (Mitchell et al., 2010; Mitchell et al., 2012; Mitchell et al., 2013; Spade et al., 2014). In addition to castration, the removal of the rat host pituitary, adrenal, and thyroid glands has been studied previously, and had no impact on time to rejection of skin xenotransplants (Bilder, 1976). Manipulation of the host should be considered as these procedures may be useful to study particular xenotransplant endocrine functions.

Responsible Conduct of Research in Xenotransplantation Studies

Xenotransplantation of human tissues into rodent hosts requires careful consideration of research ethics, as described by Panikkar et al. (2012). Acquisition of human tissue samples from living individuals must be approved by the appropriate Institutional Review Board

(IRB). Additionally, animal care, surgery, and treatment protocols must be approved by the Institutional Animal Care and Use Committee (IACUC) of the research institution(s), as applicable. Postmortem fetal tissues can be obtained as residual tissue with blanket parental consent for research or with consent for specific research projects. Researchers should consider the specific situation under which tissues are available to decide upon appropriate procedures. Laws governing fetal tissue research vary by U.S. state, as discussed in the review by Panikkar et al. (2012). Therefore, it is important for researchers to consider the requirements for obtaining consent for use of fetal tissues in research and the potential benefits and drawbacks of additional consenting procedures beyond those requirements.

The Development of Fetal Testis Xenotransplant Models for Toxicology Studies

Effects of Phthalates on the Fetal Testis and the Need for Models of Human Tissue Response

Normal development of the fetal male reproductive tract is dependent on testicular testosterone production in the Leydig cell, which can be adversely affected by environmental anti-androgens (Scott et al., 2009). In addition to Leydig cell-dependent processes, adverse effects on fetal Sertoli cells are hypothesized to contribute to the testicular dysgenesis syndrome (TDS), which links congenital disorders of the male reproductive tract with later life adverse outcomes such as testicular cancer and decreased sperm counts (Sharpe and Skakkebaek, 2008; Toppari et al., 2010). Phthalate exposure is linked to TDS (Fisher et al., 2003), but the impact of phthalates on androgen signaling in the fetal testis is subject to species differences, as described in several reviews (Johnson et al., 2012; Albert and Jegou, 2014; Habert et al., 2014a; Habert et al., 2014b). Effects of phthalates on the fetal testis have been extensively studied in the pregnant rat model, in which they cause a loss of germ cells, an increase in the number of multinucleated germ cells, and a decrease in testicular testosterone and steroidogenic gene expression, resulting in decreased masculinization of male pups (Gray et al., 2000; Mylchreest et al., 2002; Barlow and Foster, 2003; Wilson et al., 2004; Thompson et al., 2005; Ferrara et al., 2006; Howdeshell et al., 2007; Howdeshell et al., 2008; Rider et al., 2009; Hotchkiss et al., 2010; Rider et al., 2010; Hannas et al., 2011a; Hannas et al., 2011b; Hannas et al., 2012). However, a limited number of studies conducted in the pregnant mouse indicate that mouse fetal testis is relatively resistant to the anti-steroidogenic effects of phthalates or that phthalates increase testosterone at some doses in the mouse, despite similar susceptibility to germ cell effects in the two species (Gaido et al., 2007; Do et al., 2012; Heger et al., 2012; van den Driesche et al., 2012). Two other studies reported anti-androgenic effects of phthalates in fetal mice (Liu et al., 2008; Wu et al., 2010), indicating that such an effect is possible. However, it is not required for seminiferous cord effects. The differential response of the rodent models to phthalates raises the question of how accurately either model predicts the human response.

In vitro human fetal testis tissue culture systems have been developed and used to test a number of toxicants, including phthalates, in a system with high relevance to human biology, as described in a review by Habert et al. (2014b). Similar to *in utero* rodent studies,

phthalate exposure *in vitro* leads to gonocyte death in all species tested, but decreases in testicular testosterone are only consistently seen in the rat, not human or mouse tissue (Hallmark et al., 2007; Chauvigne et al., 2009; Lambrot et al., 2009; Lehraiki et al., 2009; Chauvigne et al., 2011; Muczynski et al., 2012; Habert et al., 2014b), with the notable exception of MEHP decreasing testosterone in LH-stimulated GD 18.5 mouse testis in the study by Lehraiki et al. (2009). Subsequently, human fetal testis xenotransplant models have been developed and used to test the response of the tissue to phthalates, including anti-androgenic effects and alterations in germ cells. For the fetal testis xenotransplantation model to provide physiologically relevant toxicology data, it had to be confirmed that the tissue was viable in the host and that the response to phthalate was not strongly influenced by the host.

Rodent-to-Rodent Fetal Testis Xenotransplantation

To demonstrate the feasibility of human fetal testis xenotransplantation as a model for studying environmental toxicants, we performed a series of rodent-into-rodent xenotransplantation experiments (Heger et al., 2012). C57BL/6 mouse fetal testes obtained on gestational day 15 and Fisher rat fetal testes obtained on gestational day 16 were transplanted into the renal subcapsular space of intact adult male RNU rats and BALB/c nude mice through an abdominal midline incision. Xenotransplant-bearing hosts were exposed to di-*n*-butyl phthalate (DBP) at 100, 250, or 500 mg/kg/d for one to three days, after which xenotransplanted tissue was analyzed for histopathological changes in the seminiferous cord, as well as changes in the expression of key genes (*Star*, *Scarb1*, *Cyp11b1*, and *Cyp17a1*) related to testosterone biosynthesis. Regardless of the host species, DBP treatment led to an increase in multinucleated germ cells in both rat and mouse testis, but only reduced steroidogenic gene expression levels in the transplanted rat testis (Heger et al., 2012). Results were similar to *in utero* fetal rat and mouse phthalate studies (Gaido et al., 2007), indicating that the xenotransplantation studies could accurately assess the effects of phthalates on the testis, without being heavily influenced by the host species.

Human-to-Rodent Fetal Testis Xenotransplantation

Following validation of the fetal testis xenotransplantation approach with rodent-into-rodent studies, we performed a series of human fetal testis xenotransplantation studies (Heger et al., 2012). Xenotransplantation of human fetal testis into RNU rats was conducted as for rodent-into-rodent studies. The xenotransplants were histologically normal with CD31 immunohistochemical labeling indicating that the donor vascular tissue was still present after xenotransplantation. BrdU labeling of the xenotransplants demonstrated that Sertoli cells, interstitial cells, and some germ cells were actively undergoing cell division. Exposure to DBP at 100, 250, or 500 mg/kg/d over 1 to 3 days led to an increase in the proportion of multinucleated germ cells in histological sections, consistent with both the mouse and the rat response *in utero* and in the xenotransplantation studies. However, similar to the mouse fetal testis response, DBP did not significantly alter expression of *CYP11A1*, *CYP17A1*, *SCARB1*, *STAR*, or *INSL3* after a two day exposure. A limitation of these rodent and human fetal testis xenotransplantation studies is that they were performed in an intact adult male rat host. The presence of host testosterone may have impacted measurement of gene expression endpoints

in the xenotransplants. For this reason, the model was subsequently adapted to use a castrate host, based on the approach of Mitchell et al. (2010; 2012), which is discussed below.

Effects of Anti-Androgens in Human Fetal Testis Xenotransplant Models

Richard Sharpe and colleagues developed a human fetal testis xenotransplantation model using an hCG-stimulated, castrate CD-1 nude mouse host (Mitchell et al., 2010). The authors found no impact of DBP or diethylstilbestrol on circulating testosterone or seminal vesicle weights of hosts bearing human fetal testis xenotransplants, while DBP significantly reduced the seminal vesicle weights of hosts bearing rat fetal testis xenotransplants (Mitchell et al., 2012; Mitchell et al., 2013). These results indicate that testosterone produced by the xenotransplanted fetal testis tissue can maintain the weights of the androgen-dependent secondary sex organs and that the model response depends on the species of the xenotransplanted tissue. To optimally measure the anti-androgenic effects of xenobiotics, using this approach as a guide, we adapted the model developed by Heger et al. (2012) to use an hCG-stimulated, castrate athymic nude mouse host with renal subcapsular human fetal testis xenotransplants (Spade et al., 2014). We measured circulating concentrations of graft-derived hormones in host serum, as well as the weights of three androgen-sensitive host accessory sex organs, a choice of endpoints modeled after the Hershberger assay (Gray et al., 2005). We compared the effects of a high dose of DBP with abiraterone acetate, a potent anti-androgen that irreversibly inhibits the CYP17A1 enzyme required for testosterone biosynthesis (Duc et al., 2003). In the castrate, adult athymic nude mouse host, we performed a 14-day exposure by oral gavage to a high dose of abiraterone acetate (75 mg/kg/d) or DBP (500 mg/kg/d). Abiraterone treatment reduced circulating testosterone in the xenograft model, as well as the weights of the host seminal vesicles and levator ani-bulbocavernosus muscles. DBP had no effect on these endpoints. DBP-treated xenotransplants had a slightly elevated number of multinucleated gonocytes, though this was not statistically significant (Spade et al., 2014).

Taken together, the results of these studies support the hypothesis that human fetal testis is more resistant to the anti-androgenic effects of DBP than rat fetal testis. On the other hand, germ cell effects are consistently observed following phthalate treatment, regardless of whether anti-androgenic effects are observed. This indicates that the mechanisms by which phthalates affect Leydig cells may differ from the mechanisms that affect germ and Sertoli cells; anti-androgenic effects on the Leydig cell do not appear to be required for phthalates to alter the seminiferous cord. It should be noted that the testis tissues used for these studies were not from the range of gestational ages identified as the “masculinization programming window,” when phthalate exposures may have the most severe impact on masculinization (Scott et al., 2009). Therefore, additional studies should be conducted with human fetal testis xenotransplants from earlier gestational ages to determine whether the anti-androgenic effect would differ based on gestational age. Additional future studies should be performed to determine the mechanisms underlying the germ cell effects of phthalates, as both the anti-androgenic effects and seminiferous cord effects of phthalates are putatively linked to Testicular Dysgenesis.

Human Fetal Prostate Xenotransplants and Endocrine Disruption

Prostate development is heavily influenced by sex steroid hormone signaling (McPherson et al., 2008). In addition, the etiology of prostate diseases, including benign prostatic hyperplasia and prostate cancer, involves hormone-dependent mechanisms (Taneja et al., 2004; McPherson et al., 2008; Nelles et al., 2011; Nicholson and Rieke, 2011) and may be influenced by *in utero* exposure to endocrine disruptors (Yonemura et al., 1995). In the rat, *in utero* exposure to estrogenic compounds is linked to later development of prostatic intraepithelial neoplasia (PIN) lesions, with a hypothesized epigenetic mechanism (Prins et al., 2008). However, the effects of estrogens on tissue morphology and epigenetic control of gene expression have not previously been studied in human fetal prostate tissue.

We developed a human fetal prostate xenotransplantation model to examine the mechanisms by which fetal exposure to estrogenic chemicals impacts the development of the prostate, and whether exposures have mechanistic implications for prostate disease development. A mechanistic investigation of human fetal prostate xenotransplant maturation using histological and immunohistochemical techniques has served to validate this model (Saffarini et al., 2013). Fetal prostate samples obtained from spontaneous pregnancy losses at weeks 12–24 of gestation were dissected and transplanted through a retroperitoneal incision into the renal subcapsular space of an RNU rat host. Xenotransplants were allowed to develop untreated in the host, and were harvested 7, 30, 90, and 200 days following surgery. On histological examination, the xenotransplants displayed normal prostate morphology. Development of the xenotransplants in the hosts was accelerated, as shown by caldesmon staining. Caldesmon is an adult smooth muscle marker (Walther et al., 2012) that was not present in 7-day xenotransplanted human fetal prostate samples (Saffarini et al., 2013). This likely reflects the response of fetal tissue to the hormonal environment of the intact adult male host. Transplanted tissue showed a high proliferative index and appropriate expression of both epithelial and stromal tissue markers over the first 90 days in the host. Androgen receptor and estrogen receptor alpha staining were observed throughout the experimental period, and prostate-specific antigen staining increased throughout the 200-day window, indicating normal endocrine function in the xenotransplanted tissue (Saffarini et al., 2013). We are currently working with the human fetal prostate xenotransplantation model to measure the impact of estradiol exposure on the prostate tissue, including impacts on gene expression and epigenetic control. The goal of these studies is to identify developmental processes in human prostate that are susceptible to endocrine disruption, and persistent molecular effects, such as those on the epigenome, which may contribute to later life prostate disease development.

Human Fetal Lung Xenotransplantation to Study Tissue Remodeling

In utero exposure to environmental toxicants and other adverse conditions can disrupt the development of the lung, leading to respiratory disease in children and adults (Harding and Maritz, 2012). The impact of *in utero* and early life arsenic exposure in the development of adult respiratory disease is an emerging concern (Vahter, 2008; Soto-Martinez and Sly, 2010). Therefore, we developed a human fetal lung xenotransplantation model with the goal of assessing the effects of xenobiotic exposures.

We implanted gestational week 14–22 fetal lung tissue into RNU rats or SCID Beige mice using either a subcutaneous or renal subcapsular site of transplantation and recovered the transplants after four weeks (De Paepe et al., 2012a). Although the RNU rats were more amenable to surgical procedures due to their larger size and more robust immune system, the B- and T-cell deficient and NK-cell impaired SCID Beige mouse provided a better host environment than the RNU rat. In the RNU rat, fetal lung xenotransplants were frequently targeted by inflammatory cells, which inhibited vascularization, altered morphology, and reduced viability in the xenotransplanted tissue. In renal subcapsular xenotransplants, vascular morphology and density was typical for gestational age, and vascular markers including *VEGFA*, *endoglin*, and *angiopoietin-2* were expressed more highly than in subcutaneous transplants. The subcutaneous transplants also displayed morphologically abnormal vasculature.

To determine whether fetal lung xenotransplants would produce alveolar type II cells, we implanted gestational week 13–22 human fetal lung tissue into the renal subcapsular space of RNU rats (De Paepe et al., 2012b). We were particularly interested in alveolar type II cells because they are progenitor cells with potential uses in regenerative lung medicine. The transplants matured significantly over two weeks, with a higher proliferative index than the original preimplantation tissue, including proliferation of alveolar type II cells, identified by expression of surfactant protein C. Notably, gestational week 13–17 xenotransplants expressed surfactant protein C mRNA more highly than those from gestational week 19–22, suggesting that they are a superior source of these critical regenerative cells. In future toxicological applications of this model, the alveolar type II cell population will be of interest, as type II cells are known to be sensitive to toxicants such as silica (Miller and Hook, 1990), zinc oxide (Xie et al., 2012), cigarette smoke (Messier et al., 2013), and ingested ethanol (Alli et al., 2014).

This model has shown promise for toxicological studies. In general, we have found that human fetal lung xenotransplants are viable and exhibit brisk proliferative and secretory activity (De Paepe et al., 2012a; 2012b). However, lung grafts have a mildly dysplastic appearance, reminiscent of the previously described features of fetal lungs following in utero tracheal occlusion (De Paepe et al., 1999). It is tempting to speculate that the lung xenograft phenotype may be explained by lack of efflux of secretory products, resulting in progressive distention of the airspaces. However, other non-physiologic characteristics of lung xenografts such as space constraints and lack of fetal breathing movements required for normal lung development may also play a role. Notably, human fetal lung xenotransplant models have a long history of development and use, spanning over three decades. These include subcutaneous (Cobb, 1975; Peault et al., 1994; Berin et al., 2001; Pavlovic et al., 2008; Valbuena et al., 2014) and renal subcapsular models (Peault et al., 1994; Pavlovic et al., 2008), which have been used to study normal lung, cystic fibrosis, and viral infection. Having characterized fetal lung epithelial and vascular development in this xenotransplant model, we are performing ongoing experiments to assess the impact of arsenic exposure on human fetal lung.

Human Fetal Tissue Xenotransplant Models to Study the Mechanistic Impact of Toxicants on the Development of Metabolic Syndrome

Human Fetal Liver

Epidemiologic studies have shown that adults who as neonates displayed poor growth are at higher risk for developing type 2 diabetes, cardiovascular disease, and obesity (Hales et al., 1991; Barker et al., 1993; Fernandez-Twinn and Ozanne, 2006). Nutrient restriction during pregnancy in the rodent leads to growth-retarded offspring with increased susceptibility to later life effects, similar to what has been observed in humans (Shepherd et al., 1997; Hales and Barker, 2001; Petry et al., 2001; Galjaard et al., 2013). The liver plays a critical role in whole body metabolism, and fetal liver development is affected by changes in the in utero environment caused by maternal nutrient status and exposure to environmental toxicants (Ozanne et al., 1996; States et al., 2012). We undertook the development of a xenotransplant model in order to study the effects of toxicants on fetal liver development and metabolic programming.

Transplantation studies using isolated adult and embryonic hepatocytes have shown that isolated cells quickly lose their proliferative potential and undergo apoptosis when transplanted into a quiescent liver. Furthermore, it has been demonstrated that intercellular interactions are required for the survival and function of transplanted hepatocytes (Notenboom et al., 1996; Kumaran et al., 2005; Aird, 2006). These studies suggest that transplantation of intact liver fragments that contain all liver cell types would be advantageous. Previous work on the xenotransplantation of fetal porcine liver into immunocompromised rodents supported the feasibility of human fetal liver xenotransplantation (Katchman et al., 2008).

We transplanted human fetal liver ranging in age from 14–22 gestational weeks into the renal subcapsular space of RNU rats. Xenotransplants were harvested at time points ranging from 48 hours to 2 months. Xenotransplants were characterized using histological, immunohistochemical, and gene expression analyses informed by previous extensive characterization of late gestation fetal liver development in the rodent (Sanders et al., 2012). Histological analysis of the xenotransplanted tissue at the earliest time points revealed hemorrhage and loss of hepatocytes. A number of alternative approaches were investigated, including different transport media [Leibovitz's L-15 versus V7 preservation media (Fisher et al., 1996)], animal hosts (RNU rats, NOD SCID mice, and SCID Beige mice), post-delivery interval (eight to 36 hours), transplantation sites (renal subcapsular, subcutaneous, and intraperitoneal), and both brief and extended durations following transplantation (48 hours to 2 months). In all cases, there was a rapid and profound loss of hepatocytes. It was hypothesized that the fetal hepatocytes were undergoing apoptosis due to the postmortem interval resulting in a loss of endothelial cells or changes in the interaction between hepatocytes and surrounding cells. However, it may also have been a consequence of transplantation into an environment that could not support hepatocyte viability.

Rodent studies have shown that successful engraftment and repopulation by transplanted hepatocytes requires severe injury to the host liver in order to prevent host hepatocyte

proliferation followed by a growth stimulus such as 2/3 partial hepatectomy (Sanders and Hixson, 2013). This approach requires complicated models of liver injury in which the timing of transplantation is critical. Given the spontaneous nature of the acquisition of human fetal tissue for transplantation and the fragile state of the host following xenotransplantation we did not pursue the combination of severe liver injury with xenotransplantation. In our studies, hematopoietic and biliary epithelial cells persisted in the xenotransplant, indicating the viability of the transplanted tissue. At later time points, bile ductular structures were observed, suggesting that this model may be valuable for studying human bile duct morphogenesis. However, as the experimental goal was to determine the effect of exposures on fetal metabolic programming and the development of metabolic syndrome, liver as a target tissue could not be studied using the xenotransplantation approach described here.

Human Fetal Adipose Tissue

To better understand the fetal origins of adult metabolic syndrome, obesity and diabetes, we developed a human fetal adipose tissue xenotransplant model as a complementary approach to the xenotransplantation of fetal hepatocytes. Adipogenesis in the fetus is a key determinant of postnatal adiposity and by extension risk for obesity and obesity-related diseases later in life. A number of *in vitro* and *in vivo* rodent studies have shown that environmental toxicants, such as organotins and endocrine disruptors, interact with key regulators of adipocyte differentiation, thus promoting adipogenesis (Grun, 2010). However, the mechanism of action of environmental obesogens and their impact on human fetal white adipose tissue (WAT) development and metabolism is unclear.

Preliminary rodent-to-rodent studies focused on the xenotransplantation of perigonadal fat derived from three-week old mice into the renal subcapsular space of RNU nude rats. Similar studies were performed using WAT derived from rat and transplanted into SCID-Beige mice. These studies validated the feasibility of the model and confirmed that the transplanted WAT underwent normal remodeling characterized by coordinated adipogenesis and angiogenesis resulting in healthy viable fat pads. Having established the rodent-to-rodent xenotransplantation model, human WAT was transplanted into a SCID Beige mouse host. The WAT used for transplantation was taken from the perirenal location of fetuses ranging in age from 20–22 weeks. The host mice were euthanized at time points ranging from 2 days to 6 months (Garcia et al., 2014).

Both gross and histologic examination showed that the xenotransplants underwent an early crisis period that was characterized by hemorrhage and loss of mature adipocytes. However, small foci of developing adipose tissue were apparent by 2 weeks. Obvious fat pads were present at 4 weeks and beyond (Garcia et al., 2014). Histological analysis of the fat pads showed evidence of adipogenesis (marked variability in adipocyte size) and maintenance of healthy adipocytes. Immunohistochemical staining gave evidence for active adipogenesis and angiogenesis at 4 weeks and beyond. We are currently using this model to study the effects of host nutrition and toxicant exposure on the development, gene expression, and epigenetic signature of transplanted human fetal WAT.

Advantages and Disadvantages of Xenotransplantation Models for Studying the Effects of Toxicants on Fetal Tissues

Xenotransplantation models offer a number of advantages for studying human tissue responses to xenobiotics, but also have tissue-specific limitations relating to the ability of the host to simulate the normal physiological conditions experienced *in vivo*. One such limitation is the lack of host immune cells in an immunocompromised host, which can affect the function and development of some tissues. For example, inflammatory macrophages have a role in both normal and pathological adipose tissue growth (Sun et al., 2011; Wernstedt Asterholm et al., 2014). In fetal tissue xenotransplantation, species differences in the endocrine system, the lack of a placenta, and differences in endocrine actions of fetal and adult organs such as the brain and pituitary gland may also have implications for both tissue development and response to toxicants. Therefore, optimizing endocrine factors is a major concern for organs such as the testis and prostate. Physical limitations of the host environment also exist as in the case of the lung. As noted above, the lung xenotransplants have a mildly dysplastic appearance that may be caused by abnormal physical parameters of the host environment or a lack of normal secretory products.

In a xenotransplant study, the host acts, in essence, as an *in vivo* incubator to provide physiological support to a tissue sample. Our published studies, as well as those from other researchers, indicate that many fetal tissues thrive in this environment, exhibiting normal tissue morphology and development. In the context of studying developmental origins of disease, this can offer researchers the ability to determine the mechanistic response of a tissue to a toxicant, including changes in persistent regulatory mechanisms such as DNA methylation.

It should be noted that xenotransplant hosts do not, in most cases, prematurely age the tissue sample. Prostate tissue, in an intact adult rat host appears to undergo accelerated maturation from fetal-like to adult-like tissue (Saffarini et al., 2013), but the model does not produce aged prostate tissue (i.e. morphologically, the tissue does not resemble old-age tissue). It would serve the goal of studying later life outcomes of fetal exposures to develop approaches for accelerating the aging of xenotransplanted tissues; however, no such approaches currently exist. Xenotransplant models do, however, provide an opportunity to study the mechanistic events that happen as a response to toxicant exposure in fetal tissue, including exposures over longer windows than are possible using *in vitro* organ or tissue culture.

The most significant challenge in studying the long-term effects of fetal exposures is the intractability of time. All models address this challenge through some compromise. Human fetal tissue xenotransplant models overcome a limitation by providing mechanistic data from toxicant exposures in actual human fetal tissue, which maximizes species relevance. This is complementary to the use of animal studies and epidemiological studies, which similarly offer advantages and disadvantages for studying fetal exposures. Animal models maximize the duration of exposure and outcomes relative to lifespan, and provide the distinct advantage of occurring in an intact animal, where metabolic mechanisms, endocrine pathways, and other system-level processes are integrated. Epidemiological studies provide

exposure and outcome data in humans, but without the controlled environment of a laboratory study.

Even with these limitations, xenotransplant models provide a valuable option for studying responses to toxicants in whole human fetal tissue, as the model provides human-relevant mechanistic data on toxicant response at the tissue level. The host supports the xenotransplant and metabolizes xenobiotics, while its endocrine system can be optimized to meet the needs of the xenotransplanted tissue. The resulting environment promotes normal tissue development and allows for sustained growth and maturation over longer periods of time than *in vitro* culture systems. The response of the xenotransplanted tissue to xenobiotics can be assessed using histological, cellular, and molecular endpoints, and the ability to distribute a single tissue sample among multiple hosts is an advantage for experimental design allowing for maximum sample size and the increased statistical power that derives from paired analysis. Given appropriate consideration of the goals of the study and the requirements for model development, human fetal tissue xenotransplant models have shown significant promise as models for mechanistic toxicology.

Conclusions

The human fetus undergoes critical developmental events that are uniquely sensitive to disruption by exogenous factors, including xenobiotic exposures. To understand the toxic effects of xenobiotics in humans, including effects that may only be apparent in later life, requires a combination of approaches, including epidemiological studies and experiments in traditional animal models. Human tissue xenotransplantation models are unique in that they allow for assessment of mechanistic responses to xenobiotic exposures in human tissue, an approach that can complement existing models and contribute to the understanding of impacts of fetal toxicant exposures. We are using human fetal testis xenotransplant models to investigate the histological and molecular effects of xenobiotic exposures on multiple fetal tissues, including the testis, prostate, lung, and adipose tissue. These studies will provide mechanistic data on the toxic effects of xenobiotics in developing human tissues, with the ultimate goal of characterizing persistent impacts of exposures on developmental processes that affect health and disease.

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Figure 1. Human fetal tissue xenotransplantation to study toxicology

In this example, human fetal tissue is transplanted into the renal subcapsular space of an immunocompromised rodent host. The toxicant is administered to the host through a standard exposure route such as oral gavage. The host can also be treated with hormones or other factors to support xenotransplant tissue function. In our studies, xenotransplanted tissue has been supported for up to 200 days in the host. At the conclusion of the study, the xenotransplant is collected and assessed using endpoints such as histology and gene expression. Blood samples and relevant organs can also be collected from the host for analysis.

Figure 2. Structure and function of xenotransplanted human fetal tissues in rodent hosts

Representative images of human fetal testis (A–C), prostate (D–F), lung (G–I), and adipose tissue (J–L) are shown following transplantation into the renal subcapsular space of rodent hosts. The left column shows hematoxylin and eosin-stained sections of the xenotransplanted tissue in the host kidney, including a 20 gestational week human fetal testis sample in an athymic nude mouse kidney after four weeks with human chorionic gonadotropin stimulation (A), a 20 gestational week human fetal prostate sample in an RNU rat kidney after 30 days (D), a 17 gestational week human fetal lung sample in a SCID Beige mouse kidney after 28 days (G), and a 20.6 gestational week human fetal adipose tissue sample in a SCID Beige mouse kidney after 95 days (J). Scale bars in A, D, G, and J = 1 mm. The center column shows immunohistochemical localization of human CD-31 endothelial cell marker in xenotransplants, including a 20 gestational week human fetal testis sample following 3 days in an RNU rat host (B), a 20 gestational week human fetal prostate sample following 14 days in an RNU rat host (E), a 16 gestational week human fetal lung sample following 14 days in an RNU rat host (H), and a 20.6 gestational week human fetal adipose tissue sample following 35 days in a SCID Beige mouse host (K). In H and K, human CD31 labeling can be seen extending into the host kidney. Proliferation of various cell types is demonstrated in the right hand column, including Ki-67 labeling of Sertoli, germ, and interstitial cells in a 22 gestational week human fetal testis sample, following 3 days in an RNU rat host (C), Ki-67 staining of epithelial and stromal cells of a 23.7 gestational week human fetal prostate sample following 14 days in an RNU rat host (F), Ki-67 labeling of endothelial, epithelial, and stromal cells of a 17 gestational week human fetal lung sample following 14 days in an RNU rat host (I), and immunofluorescent 5-ethynyl-2'-deoxyuridine (EdU) labeling (green) of adipocytes in a 20.6 gestational week human fetal white adipose tissue sample following 35 days in a SCID Beige mouse host, with DAPI nuclear counterstain (blue) (L). All immunohistochemical images were

counterstained with hematoxylin except panel F (methyl green). Scale bar = 50 μm for B, C, E, F, H, I, K, and L.

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