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Renal Subcapsular xenografing of human fetal external genital tissue – A new model for investigating urethral development

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ABSTRACT

In this paper, we introduce our novel renal subcapsular xenograft model for the study of human penile urethral and clitoral development. We grafted fifteen intact fetal penes and clitorides 8–11 weeks fetal age under the renal capsules of gonadectomized athymic mice. The mice were treated with a subcutaneous pellet of dihydrotestosterone (DHT), diethylstilbestrol (DES) or untreated with hormones. Xenografts were harvested after fourteen days of growth and analyzed via serial histologic sectioning and immunostaining for Ki-67, cytokeratins 6, 7 and 10, uroplakin and the androgen receptor. Non-grafted specimens of similar fetal age were sectioned and immunostained for the same antigenic markers. 14/15 (93.3%) grafts were successfully propagated and harvested. The developing urethral plate, urethral groove, tubular urethra, corporal bodies and preputial lamina were easily identifiable. These structures demonstrated robust cellularity, appropriate architecture and abundant Ki-67 expression. Expression patterns of cytokeratins 6, 7 and 10, uroplakin and the androgen receptor in xenografted specimens demonstrated characteristic male/female differences analogous to non-grafted specimens. DHT treatment reliably produced tubularization of nascent urethral and vestibular structures and male patterns of androgen receptor expression in grafts of both genetic sexes while estrogenic or hormonally absent conditions reliably resulted in a persistent open urethral/vestibular groove and female patterns of androgen receptor expression. This model's success enables further study into causal pathways by which endocrine-disrupting and endocrine-mimicking substances may directly cause disruption of normal human urethral development or hypospadias.

1. Introduction

Hypospadias is the second-most-common congenital anomaly of the urogenital tract, occurring in 1:200 to 1:300 live male births in the USA (Paulozzi et al., 1997; Paulozzi, 1999; Gallentine et al., 2001). Hypospadias is an abnormality of urethral development characterized by an ectopic urethral meatus on the ventral aspect of the penis, an abnormal urethral corpus spongiosum, penile curvature and foreskin abnormalities (Baskin, 2000; Mieusset and Soulie, 2005). Office visits, surgical treatment and hospital recovery together constitute a substantial cost to the healthcare system (Pohl et al., 2007). Patients with hypospadias seek sexual contacts and engage in sexual intercourse at lower rates than age-matched controls and are significantly more likely to report dissatisfaction with their genitals (Mieusset and Soulie, 2005). While the etiology of hypospadias remains unknown, both genetic susceptibility and environmental exposures appear to contribute (Kalfa et al., 2011; Yiee and Baskin, 2010; Hsieh et al., 2008; Choudhry et al., 2015; van der Zanden et al., 2011). Environmental exposures to estrogens, anti-androgens, and industrial and agricultural chemicals cause hypospadias in laboratory animals and have been linked to hypospadias in human epidemiologic studies (Kim et al., 2004; Kojima et al., 2002; Gray et al., 1994). Human males exposed in utero to the xenoestrogen diethylstilbestrol (DES) have an increased incidence of hypospadias (Henderson et al., 1976; Klip et al., 2002; Bibbo et al., 1977). A recent multicenter study showed a strong association between parental occupational and

Abbreviations: AR, Androgen Receptor; BPA, Bisphenol A; DES, Diethylstilbestrol; DHT, Dihydrotestosterone; H & E, Hematoxylin and Eosin; IACUC, Institutional Animal Care and Use Committee; K6, Cytokeratin 6; K7, Cytokeratin 7; K10, Cytokeratin 10; PBS, Phosphate-Buffered Saline; PCR, Polymerase Chain Reaction; SCID, Severe Combined Immunodeficiency; SRY, Sex-Determining Region Y; UCSF, University of California, San Francisco

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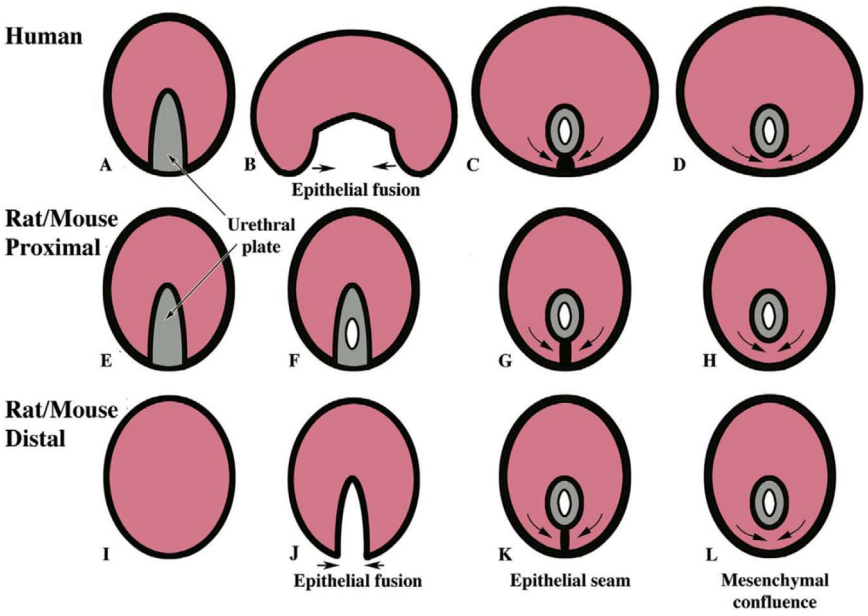

Fig. 1. Diagrammatic representations of urethral development in the human penile shaft (A–D), the human glans/rat and mouse proximal penis (E-H) and rat and mouse distal penis (I–L) showing similarities and differences. Note that the urethra in the shaft of the human penis forms by fusion of the urethral folds whereas this mechanism occurs only in the distal glans of the rat penis and mouse penis. In contrast, the urethra in the human glans forms by direct canalization of the urethral plate.

environmental exposures to endocrine disruptors such as solvents, adhesives, Bisphenol A (BPA), pesticides, phthalates and flame retardants and development of hypospadias in male children (OR 3.13 95% CI 2.11–4.65) (Kalfa et al., 2015).

The two most widely used models for the study of hypospadias in the laboratory are the mouse and rat (Cunha et al., 2015; Sinclair et al., 2016a, b; Phillips et al., 2015). Extensive efforts have focused on understanding urethral development in these species (Rodriguez et al., 2012, 2011; Weiss et al., 2012; Mahawong et al., 2014a, b; Blaschko et al., 2013). Unfortunately, rats and mice are imperfect models of human hypospadias for multiple reasons, including the presence of penile cartilage and bone (Phillips et al., 2015; Rodriguez et al., 2011), the existence of internal and external prepuces, low rates of spontaneous (non-induced) hypospadias and the absence of perineal or penile-shaft hypospadias (Cunha et al., 2015; Sinclair et al., 2016a). As morphogenic mechanisms of penile urethral development in rodents are radically different from human mechanisms (Fig. 1), it is not surprising that murine “hypospadias” is substantially different morphologically from the condition seen in humans (Cunha et al., 2015). Given these vast anatomic differences, it has become apparent that to fully understand human hypospadias, the focus must be on human urethral development. Definitive proof that environmental agents adversely affect human penile urethral development should come from direct testing of endocrine agents on human fetal organs.

There is an extensive literature on xenogransplantation of human fetal tissue into immunodeficient mice stretching back to 1974, when Povlsen et al. demonstrated successful propagation and histologic preservation of human fetal thymic, lung, pancreatic, adrenal, renal, testicular and ovarian tissue grown subcutaneously in athymic mice for 5–64 days (Povlsen et al., 1974). Since this time, researchers have demonstrated normal growth and normal differentiation of human fetal intestine (Winter et al., 1991), skin (Lane et al., 1989), lung (Groscurth and Tondury, 1982), male and female reproductive tract (Taguchi et al., 1984, 1983; Robboy et al., 1982), prostate (Sugimura et al., 1988; Yonemura et al., 1995) and ovary (Poulain et al., 2014) transplanted into athymic or severe combined immunodeficient (SCID) mice. Graft differentiation with measurable production of circulating graft-specific hormones has been demonstrated in xenografted human fetal pancreas (Usadel et al., 1980; Tuch et al., 1984; Peterson et al., 1989), pituitary (Bastert et al., 1977) and testis (Mitchell et al., 2010).

Xenotransplantation provides a powerful model to ethically evaluate the effects of therapeutics and toxicants on target human tissues. Grafted cancer cells from a wide range of primary sites demonstrate high engraftment rates, biological stability and preservation of tumor architecture and molecular markers in immunodeficient mice, allowing for evaluation and development of novel anticancer agents (Jin et al., 2010; Wang et al., 2005a). Intact xenografted human fetal internal reproductive organs have been extensively used to study the teratogenic effects of progesterone, diethylstilbestrol and other xenoestrogens on the developing prostate (Sugimura et al., 1988; Yonemura et al., 1995) and female genital tract (Robboy et al., 1982; Taguchi et al., 1983; Cunha et al., 1988). Grafted organs in these models demonstrate high engraftment rates in both control and toxicant groups under a variety of experimental conditions.

Human tissue xenografts have been successfully propagated utilizing a variety of locations within the immunocompromised mouse, including subcutaneous (Povlsen et al., 1974; Winter et al., 1991; Tuch et al., 1984), renal subcapsular (Taguchi et al., 1984; Yonemura et al., 1995; Cunha et al., 1988; Cunha and Baskin, 2016), orthotopic (Manzotti et al., 1993; Shaw et al., 2004; Wang et al., 2005b) and intraperitoneal (Shaw et al., 2004; Ward et al., 1987; Jorfi et al., 2015) sites. While orthotopic and intraperitoneal sites have advantages in promoting tumor growth and metastasis in experiments with human cancer cells, the subcutaneous and renal subcapsular sites have been more extensively utilized in xenotransplantation of intact human fetal organs. The subcutaneous site has the advantages of easy access and large surface area and provides the ability to visually and non-invasively monitor the growth of the graft, however graft take rates are low. The renal subcapsular site is more extensively vascularized, resulting in graft take rates approaching 100% (Wang et al., 2005a; Cunha and Baskin, 2016).

In the absence of a suitable animal model to study human urethral development, hypospadias and the effects of exogenous hormones, endocrine disruptors and toxicants on these processes, we have developed a novel and ethical system of investigation. In this work, we describe our renal subcapsular xenografting model for the study of urethral development utilizing human fetal penes and clitorides grown in athymic mouse hosts. We demonstrate a high rate of engraftment,