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Nonhuman primate models of polycystic ovary syndrome

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Abstract

With close genomic and phenotypic similarity to humans, nonhuman primate models provide comprehensive epigenetic mimics of polycystic ovary syndrome (PCOS), suggesting early life targeting for prevention. Fetal exposure to testosterone (T), of all nonhuman primate emulations, provides the closest PCOS-like phenotypes, with early-to-mid gestation T-exposed female rhesus monkeys exhibiting adult reproductive, endocrinological and metabolic dysfunctional traits that are co-pathologies of PCOS. Late gestational T exposure, while inducing adult ovarian hyperandrogenism and menstrual abnormalities, has less dysfunctional metabolic accompaniment. Fetal exposures to dihydrotestosterone (DHT) or diethylstilbestrol (DES) suggest androgenic and estrogenic aspects of fetal programming. Neonatal exposure to T produces no PCOS-like outcome, while continuous T treatment of juvenile females causes precocious weight gain and early menarche (high T), or high LH and weight gain (moderate T). Acute T exposure of adult females generates polyfollicular ovaries, while chronic T exposure induces subtle menstrual irregularities without metabolic dysfunction.

Keywords

fetal programming; epigenome; monkey; metabolic syndrome; androgen excess

1. Introduction

Polycystic ovary syndrome (PCOS) afflicts 15% of women in their reproductive years (Fauser et al., 2012), increasing a woman's lifetime risk of type 2 diabetes mellitus (type 2

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DM) and cardiovascular disease (Wild et al., 2010). Progress towards prevention of PCOS, however, has been hindered by an incomplete knowledge of its pathogenesis. Polycystic ovary syndrome is characterized by at least two of the following criteria: hyperandrogenism, intermittent or absent menstrual cycles (often accompanied by luteinizing hormone [LH] excess), and increased numbers of small ovarian antral follicles (Fauser et al., 2012). While a hyperandrogenic polycystic ovary is central to PCOS pathophysiology (Gilling-Smith et al., 1997; Franks et al., 2008; Nelson et al, 1999), visceral obesity and insulin resistance are frequent co-morbidities (Ehrmann et al, 2006; Kiddy et l, 1990). Pre- or peri-pubertal metabolic dysfunction is one of the first phenotypic traits observed in adolescent girls likely to develop PCOS (Coviello et al., 2006; Marshall, 2006), an alarming finding since obesity now affects ~15% of American children (Dietz, 1998; Hedley et al., 2004). Overweight or obese adolescent girls have higher circulating androgen levels than their lean counterparts (Ibanez et al., 2003; Chhabra et al., 2005; McCartney et al., 2006), with such hyperandrogenism predisposing adolescents to PCOS (Rosenfield, 2007; Zumoff et al., 1983; McCartney, 2010).

Twin (Vink et al., 2006) and genetic (Goodarzi et al., 2003; Urbanek et al, 2007; Ewens et al., 2010) studies demonstrate high heritability of PCOS, particularly hyperandrogenism (Legro et al., 1998). The most reliable PCOS gene candidate is a member of the TGF-β superfamily encoding for the extra-cellular matrix protein, fibrillin 3 (Ewens et al., 2010). The allelic fibrillin variant, A8, is linked with PCOS and manifests a distinct metabolic phenotype, even in immediate male kin, which includes insulin resistance (Urbanek et al., 2007). Circulating levels of the TGF-β superfamily peptide, anti-mullerian hormone (AMH), are derived from ovarian granulosa cells and are elevated in women and adolescents with PCOS (Pellatt et al., 2010; Siow et al., 2005; Hart et al., 2010), as well as in infant girls born to women with PCOS (Sir-Petermann et al., 2012; Crisosto et al., 2012). As daughters born to PCOS women are at increased risk for PCOS in adulthood (Vink et al., 2006), their contribution to evaluation of PCOS antecedents is relevant, particularly because reproductive and metabolic features of PCOS are established in these young girls before signs and symptoms fully manifest in adulthood (Rosenfield, 2007; Franks, 2002; Shayya and Chang, 2010; Roe and Dokras, 2011).

Consistent with an early onset of PCOS pathophysiology, aspects of PCOS-like phenotypes are reliably produced in females rhesus monkeys (Abbott et al., 2002, 2005), sheep (Padmanabhan and Viega-Lopez, 2011, this issue), and rats and mice (Walters et al., 2012; McNeilly, this issue) by *in utero* exposure to fetal male levels of testosterone (T), suggesting that the intrauterine environment may fundamentally affect the etiology of PCOS in such prenatal androgenized (PA) females (Abbott et al., 2008). Furthermore, adult female macaques have been identified with naturally occurring PCOS-like traits in which high T levels may be heritable (Airfin et al., 2008; DH Abbott, unpublished results). Consequently, fetal T programming of PCOS-like reproductive traits has been formulated as the fetal origins of PCOS hypothesis (Abbott et al., 2002), but the underlying cellular and molecular mechanisms are still poorly understood.

This review will thus examine nonhuman primate contributions to animal models for PCOS, focusing on reproductive, endocrinological and metabolic phenotypes generated by T or other steroids throughout life and the insights regarding PCOS pathogenesis such experimental manipulations provide. As rhesus monkeys share ~93% of their genome with humans (Gibbs et al., 2007), their human-like reproductive (Kaplan et al., 2010), metabolic (Zhang et al., 2011), developmental (Trounson and Grieshammer, 2012) and aging (Colman et al., 2009) traits provide readily translatable outcomes for human disease.

2. Reproductive phenotypic traits

2.1.1. *In utero* Exogenous T: Programming for PCOS in Adult Female Rhesus Monkeys

2.1.1.1. PCOS-like phenotypes: Adult female rhesus monkeys previously exposed to fetal male levels of T during early-to-mid gestation provide the most comprehensive epigenetic mimic of PCOS in women (Abbott et al., 2005, 2009; Xu et al., 2011). Fetal male T levels are achieved in fetal female monkeys by means of daily subcutaneous (s.c.) injections of their dams with 10–15 mg testosterone propionate (TP) for 15–40 consecutive days between 40 and 80 days of gestation (early-to-mid gestation T exposure, E) or for 15–25 consecutive days between 94–140 days of gestation (late gestation T exposure, L). Such T treatments raise circulating T levels in monkey dams to those found in adult males during their nocturnal peak (Dubey et al., 1984), exceed the binding capacity of circulating sex hormone binding globulin (SHBG), as well as placental ability to aromatize and hepatic ability to inactivate and conjugate T and aromatized metabolites; and creating fetal male T levels in female offspring (Resko et al., 1987; Abbott et al., 2008a) without simultaneous increases in maternal or fetal estradiol concentrations (Abbott et al., 2008a). While T is an aromatizable androgen, an increase in circulating levels of estrogens is at least partly assuaged by increased hepatic conjugation (Abbott et al., 2008a).

When adults, both EPA and LPA female monkeys exhibit ovarian hyperandrogenism (Eisner et al, 2002; Abbott et al., 2005) and intermittent or absent menstrual cycles (Abbott et al., 1998; 2005). Increased adiposity exaggerates menstrual cycle irregularity in PA monkeys (Abbott et al., 1998), as it does in exaggerating the PCOS traits of women (Wild et al., 2010). When EPA and LPA monkeys are combined, about 40% have enlarged polycystic ovaries (Abbott et al., 2009; Xu et al, 2011). Regarding nonhuman primate equivalents to the diagnostic criteria for PCOS in women (Abbott et al., 2009; Xu et al., 2011), ~67% of EPA monkeys have an identifiable PCOS phenotype, as do ~55% of LPA monkeys, with diversity of PCOS-like phenotypes in EPA monkeys resembling that of PCOS women. Approximately 50% of EPA female monkeys exhibit the severe PCOS phenotype of hyperandrogenism (circulating testosterone ≥1 SD of the control mean) and intermittent or absent menstrual cycles (>34 days), with or without a polyfollicular ovary (>10, ~1mm diameter follicles/ovary), an important health care consideration since hyperandrogenic PCOS phenotypes are more metabolically compromised from increased abdominal adiposity and insulin resistance as risk factors for cardiovascular disease and type 2 DM (Moran and Teede, 2009; Wild et al., 2010). About 8% of EPA monkeys show an ovulatory PCOS phenotype (hyperandrogenism, polyfollicular ovary and regular menstrual cycles), while ~17% have a normo-androgenic phenotype (normal testosterone levels <1SD of the control mean, polyfollicular ovary and intermittent/absent menstrual cycles) (data combined from previous studies: Abbott et al., 2009; Xu et al., 2011; Abbott DH, unpublished results), although the metabolic similarlties of these phenotypes to those of PCOS women (Wild et al., 2010; Fauser et al., 2012) remain unclear.

2.1.1.2. Accompanying Abnormalities in Reproductive Neuroendocrine Function: In addition to emulating human PCOS diagnostic criteria, EPA, but not LPA, monkeys also mimic most PCOS women by hypersecreting pituitary LH, including basal levels (Abbott et al., 1998, 2005; Dumesic et al., 1997), increased episodic release (from enhanced endogenous hypothalamic gonadotropin releasing-hormone [GnRH] secretion) (Levine et al., 2005), increased pituitary responsiveness to exogenous GnRH (Abbott et al., 2005), diminished steroid negative feedback (Steiner et al., 1976; Dumesic et al., 2002; Levine et al., 2005), and increased amplitude or bioactivity of the midcycle LH surge (Steiner et al., 1976; Dumesic et al., 1997). These LH-related traits may reflect estrogen resistance,

particularly since diminished estradiol negative (Sarma et al., 2005) and positive (Foecking et al., 2005) feedback regulation of LH secretion accompany diminished estradiol action in the hypothalamus (Robinson et al., 2010), as well as reduced hypothalamic expression of progesterone receptor (Foecking et al., 2005).

2.1.1.3. Accompanying Abnormalities in Oocytes: At the ovarian level, ovarian stimulation of adult female rhesus monkeys with gonadotrpins for *in vitro* fertilization (IVF) reveals impaired oocyte developmental competence in both groups of PA monkeys that is especially pronounced in EPA monkeys (Dumesic et al., 2002, 2003). As EPA females age, ovarian deficits increase in terms of diminished oocyte developmental competence and low circulating AMH levels, suggestive of diminished ovarian reserve (Dumesic et al., 2009). The initial IVF data from our PCOS-like nonhuman primates (Dumesic et al., 2002, 2003) led to subsequent clinical IVF studies of PCOS women (Wood et al., 2007), in which normal appearing mature (metaphase II) PCOS oocytes exhibited altered gene transcription regulation, with a subset of these genes associated with chromosome alignment and segregation, suggesting that dysregulation of the respective proteins may negatively affect oocyte maturation and/or early embryonic development. The IVF studies of EPA monkeys, unlike those of PCOS women, however, indicated diminished, rather than enhanced, ovarian responsiveness to gonadotropin therapy, as evidenced by decreased, rather than increased, numbers of growing antral follicles, as is typical of PCOS women (Heijinen et al., 2006). Thus, at the ovarian follicular level, EPA monkeys diverge from their PCOS human counterparts in their ovarian responsiveness to exogenous gonadotropins (Dumesic et al., 2002, 2003, 2009), perhaps from a genetic human abnormality (i.e., fibrillin gene variant), that alters the extra-cellular matrix of the PCOS ovary to perturb its morphology and function (Hatziridos et al., 2011; Abbott, 2012).

2.1.1.4. Evidence for Fetal Origins of PCOS in Women: Some, but not all, recent human studies are consistent with such *in utero* T exposure as a developmental programming model for reproductive and endocrine traits of PCOS. Umbilical cord blood androgen levels in one study are not elevated in adolescent girls later diagnosed with PCOS (Hickey et al., 2009). Subsequent studies on the same cohort, however, positively correlate mid-gestational maternal serum total T levels with adolescent daughter AMH serum concentrations (Hart et al., 2010). Since high circulating levels of AMH are characteristic of adolescents and adult women with PCOS (Sir Petermann et al., 2009), as well as infant girls born to PCOS women (Sir-Petermann et al., 2012; Crisosto et al., 2012), the inter-generational association invokes a potential relationship between maternal gestational hyperandrogenism and development of PCOS in adolescent daughters. Primate, including human, placental aromatization and hepatic inactivation of androgens should normally prevent fetal exposure to maternal androgen excess unless it reaches testosterone levels characteristic of testicular function (Abbott et al., 2008a), but subtle alterations in placental function in women with PCOS may compromise protection of their fetal daughters (Crisosto et al., 2012). Altered placental morphology also occurs in term placentae from EPA monkeys (Abbott DH, unpublished results) and in placentae from T-exposed ewes (Padmanabhan and Viega-Lopez, this issue).

In perinatal human studies, one finding shows diminished umbilical cord blood androstenedione levels (Anderson et al., 2010), while another notes elevated T levels in umbilical venous blood of daughters born to PCOS women (Barry et al., 2010). A study of human fetal blood obtained via cordocentesis, moreover, finds that ~40% of female fetuses at mid-gestation have T levels in the fetal male range (Beck-Peccoz et al., 1991). Further studies are needed to quantify serum androgen levels in the mid-gestational human female fetus, which may itself be the source of androgen excess *in utero* during a critical time of target tissue differentiation (Abbott et al., 2002). Agreeing with this, two studies of women with PCOS (Lujan, 2010a, 2010b) show positive correlations between adult T levels or

hirsutism scores and the finger length ratio between the 2nd and 4th fingers, an anthropometric trait that is established *in utero* (McIntyre, 2006). EPA monkeys demonstrate analogous positive correlations between the same finger length ratio and duration of T exposure during early-to-mid gestation, as well as anogenital distance (a fetal T dependent trait), suggesting that finger length associations in PCOS women may belie fetal T exposure origins (Abbott et al., 2012). EPA monkeys may thus enable focused studies of intrauterine T programming of PCOS trait development, an investigative path that is technically and ethically difficult to follow in humans.

2.1.2. *In utero* Exogenous T: Fetal and Infant Antecedents To Adult PCOS-like Reproductive Traits in Monkeys—

EPA infant monkeys are hyperandrogenic. Circulating levels of androstenedione are elevated on postnatal days 1 and 30 (Abbott et al., 2008a), along with T on postnatal day 1 (T levels in all EPA infants equal or exceed the maximum T level in newborn controls; Xu et al., 2011). Fetal hypergonadotropism, however, manifests before infant hyperandrogensim. Fetal EPA female monkeys exhibit elevated basal LH and FSH levels by 120 days of gestation (Abbott et al., 2008a). LH hypergonadotropism persists into early infancy (there are no infant data for FSH). Ovaries of infant EPA monkeys do not have a polyfollicular phenotype, but growing follicles comprise a diminished proportion of total follicles (Abbott et al., 2008b). Infant female EPA monkeys thus show early signs of ovarian and gonadotropic dysfunction. Infant daughters born to women with PCOS also exhibit early signs of ovarian dysfunction, but unlike EPA infant monkeys, the human infants have high levels of estradiol and AMH (Sir Peterman et al., 2006, 2012). Exaggerated adrenarche, a pre-PCOS trait (Rosenfield, 2007; Idkowiak et al., 2011), may contribute to infant hyperandrogenism in EPA rhesus monkeys, particularly because adrenarche occurs during early infancy in rhesus monkeys (Conley et al, 2011, 2012), with EPA monkeys, as adults, exhibiting adrenal androgen excess (Zhou et al., 2005; Abbott and Bird, 2009).

2.2. *In utero* Exposure of Fetal Female Rhesus Monkeys to Exogenous Dihydrotestosterone (DHT) or Diethylstilbestrol (DES)

In monkeys, unlike sheep (Padmanabhan and Viega-Lopez, this issue), there is little known about the relative contributions of androgen and estrogen receptors to *in utero* PCOS-like trait development. Female monkey fetuses exposed to DHT when their dams received daily s.c. injections of 10–15 mg DHTP for 55–70 consecutive days starting on days 40–45 of gestation received somewhat of an analogous in utero androgen exposure to their T-exposed EPA counterparts, as DHT exposure commenced during early gestation. DHT, however, is a non-aromatizable androgen. DHT-exposed female monkeys show a ~6-month delay in their age at menarche and adolescent onset of irregular menstrual cycles (Goy et al., 1988; Goy and Robinson, 1982). In contrast, female fetuses exposed to DES during early-to-late gestation, when their dams received daily oral doses of 1mg/kg or 1mg DES for at least 125 consecutive days starting on days 21–25 of gestation, exhibit regular menstrual cycles in adulthood, but show postnatal elevations in basal LH levels and reduced fertility from timed-mating with males (Hendrickx et al., 1988; Fuller et al., 1981). Onset of menstrual cycle dysfunction thus appears to be more of an androgen-mediated effect in monkeys, while dysfunctional regulation of LH and fertility appears to be at least partly estrogen-mediated. The steroid-specific functional changes appear in contrast to those found following *in utero* exposure in ewes in which ovarian cycle defects appear estrogen-mediated, while altered regulation of pituitary LH appears androgen-mediated (Padmanabhan and Viega-Lopez, this issue). While these fetal programming differences between PA monkeys and PA sheep may be species-specific, or dose, route of administration or gestational age-related, female fetal exposure to exogenous T in monkeys does not include elevated circulating levels of estradiol or estrone in maternal or fetal

circulations (Abbott et al., 2008). This contrasts with sheep, in which fetal estradiol and estrone are both elevated in maternal and fetal circulations during exogenous T treatment (Padmanabhan and Viega-Lopez, 2011; Hogg et al., 2011; Padmanabhan and Viega-Lopez, this issue). Increased conjugation, and thus inactivation, of circulating estrogens may diminish hyper-estrogenic consequences of fetal hyperandrogenism in primates (Abbott et al., 2008a). However, as the primate placenta and many primate fetal tissues express steroid hormone sulfo-conjugate cell membrane transporters (Ugele et al., 2003, 2008) and sulfatases (Dawson, 2011), *in utero* T-exposed female monkeys may be able to utilize increased circulating concentrations of estrogen conjugates (Abbott et al., 2008a) to generate increased intra-cellular estrogen concentrations during fetal T exposure.

2.3. Neonatal Female Rhesus Monkey Exposure to Exogenous T

No reproductive pathophysiological outcomes follow either a single s.c. injection of 1 mg TP to infant female rhesus monkeys on postnatal days 1–4 (Treloar et al., 1972) or s.c. implantation of infant marmoset monkeys with 25mg T for ~50 days commencing on postnatal days 1–4 (Abbott and Hearn, 1978; Abbott, 1984).

2.4. Juvenile Female Rhesus Monkey Exposure to Exogenous T

Daily injections of 2 mg/kg T propionate administered to 6-month old juvenile female rhesus monkeys induces accelerated weight gain and premature menarche (van Wagenen, 1949), while a high calorie diet initiated at 12 months of age also induces comparable weight gain and precocious menarche in otherwise untreated female rhesus monkeys (Terasawa et al., 2012). Thus, prolonged juvenile exposure to excess T or caloric intake have profound effects on developing female monkey reproductive maturation. In comparison, s.c. implantation of pre-pubertal female rhesus monkeys with T-filled capsules, resulting in moderate (~3.7 fold) increases in circulating T levels, does not alter puberty or species-typical adolescent menstrual irregularity, but does increase frequency of LH pulses by early adulthood, with a trend towards diminished dominant follicle selection (McGee et al., 2012). Postnatal, prepubertal onset of T excess in female monkeys may thus be able to contribute PCOS-like reproductive traits without prior fetal T exposure. Importantly, no studies of these pre-pubertal onset treatments have been performed beyond the duration of exogenous T exposure so it is not yet established whether endogenous PCOS-like traits are permanently induced by pre-pubertal onset of exogenous T excess.

Peri-pubertal origins for PCOS have been proposed from human studies demonstrating an association between peri-pubertal hyperandrogenism and increased frequency of pituitary LH pulses (McCartney, 2010). Obesity commonly accompanies peri-pubertal hyperandrogenemia in girls (McCartney et al., 2006, 2007; Blank et al, 2006, 2007), possibly representing an antecedent to PCOS (Rosenfield, 2007; McCartney, 2010). Such androgen-associated LH hypersecretion involves androgen-dependent diminished sensitivity to progesterone/estrogen-mediated negative feedback (Blank et al, 2009), an aspect of estrogen resistance. In this regard, as peri-pubertal female rhesus monkeys chronically treated with T show increased frequency of LH release (McGee et al., 2012), thereby emulating findings of hyperandrogenic adolescent girls, hyperandrogenism accompanying obesity in human adolescents may program estrogen resistance, causing a PCOS-like neuroendocrine abnormality with metabolic dysfunction that may develop into PCOS.

2.5. Adult Female Rhesus Monkeys Chronically Exposed to Exogenous T, Androstenedione or IGF-1

Subcutaneous implantation of pellets containing 20ug/kg T or 145 ug/kg DHT administered to adult female rhesus monkeys for five consecutive days, or 4 mg/kg T pellets implanted for 3 days or 400 ug/kg T pellets implanted for 10 days, all enhance ovarian follicle

recruitment, including exaggerated commitment of primordial follicles to growth (Vendola et al., 1998, 1999a). The polyfollicular ovarian phenotype of overly numerous, small-to-medium sized antral follicles is androgen receptor mediated and includes upregulated IGF-1-mediated antral follicle growth (Vendola et al., 1999a,b; Weil et al., 1999). Acute elevations of circulating T concentrations from exogenous T treatment can thus rapidly transform normal primate ovaries into polyfollicular phenotypes. In contrast, 13–16 months of exposure to 10–25 mg T s.c. capsule implants (Faiman et al., 1988) moderately elevating circulating T levels to 0.8–1.2 ng/ml, or ~12 months of androstenedione s.c. capsule implants (Billiar et al., 1985), both induce only subtle ovarian follicular abnormalities in adult female rhesus monkeys and a modest increase in intermittent and absent menstrual cycles.

Administration of a GnRH antagonist to remove endogenous gonadotropic stimulation of ovary-intact adult female rhesus monkeys was employed to examine the effects of chronic treatment with T, DHT or IGF-1 on ovarian responses to exogenous FSH and LH (Zeleznik et al., 2002, 2004). Exogenous T, DHT and IGF-1 all failed to augment exogenous gonadotropin-induced ovarian androgenic responses, while both DHT and IGF-1 diminished gonadotropin-induced ovarian estrogen responses. Taken together, female monkey studies employing adult onset elevations in exogenous androgens or IGF-1 do not appear to induce PCOS-like reproductive traits.

3. Metabolic Phenotypic Traits

3.1.1. *In utero* Exogenous T: Fetal Programming for PCOS-like Metabolic Dysfunction in Female Monkeys—EPA fetal monkeys develop many PCOS-like metabolic traits as adults (Abbott et al., 2005, 2009; Dumesic et al., 2005; Zhou et al., 2007a), including increased visceral and total abdominal adiposity, hyperlipidemia, insulin resistance, impaired insulin secretion, hyperglycemia and type 2 DM. Emulating PCOS women, most EPA monkeys respond to six months of daily treatment with the thiazolidinedione insulin sensitizer, pioglitazone, by decreasing insulin resistance and fasted basal glucose levels (Zhou et al., 2007a). Both pioglitazone-induced changes are likely contributors to EPA monkey normalization of glycosylated hemoglobin, a summation measure of circulatory glucose during the preceding 2–3 months. A recent pilot study of EPA monkey pancreatic morphology suggests diminished beta cell mass accompanying lack of pancreatic compensation for insulin resistance (Nicol et al., 2009). EPA monkey metabolic dysfunction thus closely resembles pre-diabetic and diabetic presentations commonly found in women with PCOS (Moran and Teede, 2009; Wild et al., 2010). The discrete fetal programming contributions of androgenic-, estrogenic- and hyperglycemic-mediated action to each aspect of adult EPA monkey metabolic dysfunction are unknown.

Impaired insulin action in EPA adult monkeys, however, in addition to associating with measures of hyperglycemia and increased type 2 DM, contributes to the mechanism of anovulation. During six months of daily pioglitazone treatment, ovulatory menstrual cycle duration was normalized in EPA monkeys (Zhou et al., 2007a). All pioglitazone-treated females, PA and control monkeys combined, exhibited a more estrogenic than androgenic milieu. As the converse treatment, six months of daily insulin injections, normalized follicular phase duration in EPA monkey ovulatory menstrual cycles, while diminishing circulating basal glucose levels (Zhou et al., 2007b), improvements in insulin action rather than diminished circulating insulin levels appear to be key in normalizing ovarian responses to endogenous gonadotropins in EPA monkeys. As chronic insulin therapy administered to relatively insulin sensitive humans risks episodes of hypoglycemia, chronic insulin treatment of EPA monkeys provides unique insight.

3.1.2. *In utero* Exogenous T: Fetal and Infant Antecedents To Adult PCOS-like Metabolic Dysfunction in Female Monkeys—

TP injections of rhesus monkey dams during early-to-mid gestation transiently accelerate maternal weight gain and induce modest increases in growth in their EPA female fetuses and infants (Abbott et al., 2010). Mild to moderate glucose intolerance, with increased area-under-the-curve circulating insulin values, occurs in TP-injected dams during an intravenous glucose tolerance test at mid-gestation, while reduced circulating FFA levels are found in EPA fetuses during a intravenous glucagon-tolbutamide challenge at 140 days of gestation. Interestingly, the more adipose the dams prior to conception, the more susceptible they are to TP-induced reduction in insulin regulation of glucose during pregnancy with its fetal sequelae (Abbott et al., 2010). The EPA monkey *in utero* scenario thus appears different to that experienced by T-exposed female ewes and rats, as the latter exhibit fetal growth restriction (Mannikam et al., 2004; Slob et al., 1983), the converse of EPA monkeys.

In EPA infants, excessive insulin sensitivity and increased insulin secretion relative to insulin sensitivity accompany more rapid glucose clearance during an intravenous glucose-tolbutamide test administered at 1.5 months postnatal age. By 2 months of postnatal age, EPA infant pancreatic beta cell proliferation is approximately doubled (Nicol et al., 2009; LE Nicol, unpublished results). These studies suggest that experimentally induced fetal T excess results in transient hyperglycemic episodes in the intrauterine environment that are sufficient to induce relative increases in pancreatic function in EPA infants. Such developmental changes suggest that differential programming of insulin action and secretion precede adult metabolic dysfunction in the EPA monkey model.

3.2. *In utero* Exposure of Fetal Female Rhesus Monkeys to Exogenous Dihydrotestosterone (DHT) or Diethylstilbestrol (DES)

Metabolic parameters were not assessed following fetal exposure of female rhesus monkeys to exogenous DHT or DES.

3.3. Neonatal Female Rhesus Monkey Exposure to Exogenous T

Metabolic parameters were not assessed following neonatal exposure of female rhesus monkeys to exogenous T.

3.4. Juvenile Female Rhesus Monkey Exposure to Exogenous T

Chronic high T treatment results in accelerated growth and weight gain (van Wagenen, 1949) accompanied by early menarche. In a separate study, chronic T treatment causing moderately increased circulating T levels eventually promote increased body weight by early adulthood (McGee et al., 2012). A trend towards hyperinsulinemia and insulin resistance occurs in the latter monkeys. Postnatal, pre-pubertal onset of T exposure does not induce pronounced metabolic dysfunction in ovary-intact female rhesus monkeys.

3.5. Adult Female Rhesus Monkeys Chronically Exposed to Exogenous T or Androstenedione

Adult onset of chronic T or androstenedione treatments have no obvious adverse effects on female monkey metabolic function and no demonstrable impairment of glucoregulation (Billiar et al., 1987). Adult female rhesus monkeys, therefore, appear relatively unresponsive to extant androgen-mediated induction of PCOS-like metabolic dysfunction.

4. Molecular Epigenetic Signature of Fetal T Exposure in Female Monkeys

Alteration of the epigenome is a mechanism whereby gestational T excess (Xu et al., 2011; Auger et al., 2011; Hogg et al., 2012), or its hyperglycemic consequences (Pirola et al.,

2010, 2011), reprogram gene expression in EPA monkeys enabling development of PCOS-like traits in adulthood. Differentially methylated genes identified in EPA infant (>100) and adult (>300) visceral fat are involved in the regulation of adipogenesis, intermediate metabolism and cell proliferation. Bioinformatics-based analyses identified TGF-β signaling as the most significantly perturbed pathway (Xu et al., 2011). As TGF-β signaling is currently implicated in the pathogenesis of PCOS in women by genetic epidemiologic evidence (Jones et al., 2007; Raja-Khan et al., 2010; Urbanek et al., 1999, 2007), our EPA monkey data support the potential contributory role of epigenomic perturbation in the etiology of PCOS. In support of this, since a reliable genetic marker for PCOS may be fibrillin-3 (Yalamanchi et al., 2012), a key TGF-β-related signaling element expressed in the human fetal ovary (Hatzirodos et al., 2010) when fetal T exposure most comprehensively induces PCOS-like traits in EPA female monkeys, EPA monkeys may epigenetically mimic PCOS in women. Given dysfunctional TGF-β signaling as a putative genetically-based mechanism for developing PCOS, our findings of altered methylation of TGFβ3, TGFBR1, KRAS, BMP2, TFE3, Runx3 and Hoxc8, complement previous studies implicating TGF-β superfamily members or their regulators in the pathophysiology of PCOS, including AMH, inhibin B, growth differentiation factor 9, activin A, follistatin, and fibrillin-3 (Xu et al., 2011). As fibrillins interact with latent TGF-β binding protein, and both fibrillins and follistatin share TGF-β binding domains, both regulate extracellular TGF-β bioactivity. Altered TGF-β determined extra-cellular matrix may thus contribute to altered adipogenesis, as well as stroma hypertrophy and hyperandrogensim in the ovaries of both PCOS women and EPA monkeys, particularly as hyperglycemia and hyperinsulinemia alter epigenetic regulation of fibrillin-1 and thus TGF-β signaling (Gaikwad et al., 2010). With close replication of PCOS by EPA monkeys, and as rhesus monkeys share ~93% of their genome with humans (Gibbs et al., 2007), their PCOS-like traits provides unparalleled opportunities to explore EPA monkey epigenetic molecular mimickry of the human syndrome.

5. Clinical Significance for PCOS of In Utero T Induction of PCOS-like Phenotypes in Monkeys

With the certainty of a PCOS-like phenotype in adulthood. EPA monkeys provide opportunities to determine fetal, infant and juvenile antecedents of PCOS that will enable early targeting of amelioration or prevention in humans. In addition, as TP injections given to pregnant monkey dams impair the dams' abilities to regulate blood glucose, as in humans, subsequent maternal hyperglycemia results in increased fetal exposure to glucose and increased fetal and neonatal growth. Infant EPA female monkeys previously exposed to T and high glucose exhibit high insulin responses to glucose that will likely cause insulin-induced accumulation of fat and muscle and relatively fat female juveniles. Since EPA infants are also hyperandrogenic, such additional anabolic signaling may further enhance postnatal weight gain, potentially enabling EPA female monkeys to resemble overweight, hyperandrogenic adolescent girls predisposed to PCOS in adulthood (Rosenfield, 2007; McCartney et al., 2010). These findings encourage clinical studies aimed at establishing childhood biomarkers for increased adiposity and subsequent adult PCOS, especially since PCOS mothers taking the insulin sensitizer metformin before and during pregnancy give birth to daughters who do not go on to develop ovarian hormonal abnormalities at 2–3 months of age (Crisosto et al., 2012).

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Highlights

- Early-to-mid gestation testosterone excess recapitulates PCOS phenotypes in monkeys
- Transient maternal and fetal hyperglycemia accompany testosterone exposure
- Epigenetic changes in visceral fat implicate altered TGF-β signaling
- T-exposed monkeys may provide a close, epigenetic molecular mimic of PCOS