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Selective deletion of *Pten* in theca-interstitial cells leads to androgen excess and ovarian dysfunction in mice

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ABSTRACT

Theca cell-selective *Pten* mutation (*tPtenMT*) in mice resulted in increases in PDK1 and Akt phosphorylation, indicating an over-activation of PI3K signaling in the ovaries. These mice displayed elevated androgen levels, ovary enlargement, antral follicle accumulation, early fertility loss and increased expression of *Lhcgr* and genes that are crucial to androgenesis. These abnormalities were partially reversed by treatments of PI3K or Akt inhibitor. LH actions in *Pten* deficient theca cells were potentiated. The phosphorylation of Foxo1 was increased, while the binding of Foxo1 to forkhead response elements in the *Lhcgr* promoter was reduced in *tPtenMT* theca cells, implying a mechanism by which PI3K/Akt-induced upregulation of *Lhcgr* in theca cells might be mediated by reducing the inhibitory effect of Foxo1 on the *Lhcgr* promoter. The phenotype of *tPtenMT* females is reminiscent of human PCOS and suggests that dysregulated PI3K cascade in theca cells may be involved in certain types of PCOS pathogenesis.

1. Introduction

Ovarian theca cells differentiated from interstitial cells produce androgens and growth factors to modulate follicular development (Magoffin, 2005; Wickenheisser et al., 2006; Edson et al., 2009; Orisaka et al., 2009; Young and McNeilly, 2010). They are the major source of androgens in females (Abbott et al., 2002). Hyperactivity of theca cells can cause female infertility due to hyperandrogenism (Magoffin, 2005). It is well established that androgenesis in theca interstitial (TI) cells is principally regulated by luteinizing hormone (LH). Upon the binding of LH to its receptor LHCGR activates multiple signaling pathways including the cAMP/protein kinase A and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signal cascades to influence steroidogenesis (Strauss et al., 2002; Richards et al., 2002; Palaniappan and Menon, 2010; Narayan, 2015). However, the *in vivo* function of the PI3K signaling pathway in the regulation of androgenesis in TI cells remains to be further characterized.

PI3K signaling is a complex pathway in which PI3Ks are activated by receptor-tyrosine kinases and/or G-protein-coupled-receptors (Ascoli et al., 2002; Engelman et al., 2006; Vanhaesebroeck et al., 2010a; Kriplani et al., 2015), then phosphorylate PDK1 (3-phosphoinositide-dependent protein kinase 1), subsequently Akt followed by mTOR (mammalian target of rapamycin), Foxo (Forkhead box O) 1/3/4/6, and/or GSK3 (glycogen synthase kinase-3)-α/β signaling molecules to elicit a range of biological effects, including cell growth and survival, angiogenesis and embryonic development. Phosphatase and tensin homolog (Pten) is an endogenous inhibitor of PI3K signaling by dephosphorylating a potent activator of PDK1, phosphatidylinositol-triphosphate (PIP3). Loss of Pten causes increased conversion of PIP2 into PIP3 and increased PDK phosphorylation, which stimulates Akt activity (Kriplani et al., 2015; Pulido, 2015; Milella et al., 2015).

Pten is encoded by a gene located on chromosome 10q23 in humans and on chromosome 19 in mice and is expressed in all

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types of ovarian cells (Milella et al., 2015; Carracedo and Pandolfi, 2008; Fan et al., 2008; Salmena et al., 2008). In order to determine the significance of Pten in the ovary, several groups have performed studies in this area. Reddy et al. created a mouse model with selective Pten knockout only in the oocytes (Reddy et al., 2008; Liu et al., 2015). This model showed early activation of follicular development with resulting premature ovarian failure. Fan et al. were able to selectively delete *Pten* in murine granulosa cells, with resulting increase in ovulation and a moderately increased number of pups, as well as prolonged persistence of corpora lutea (Fan et al., 2008). However, the role of Pten in TI cells remains elusive.

To explore the function of PI3K signaling in TI cells *in vivo*, we generated selective TI cell *Pten* mutant (*tPtenMT*) mice. Deletion of *Pten* in TI cells leads to over-activation of PI3K signaling, androgen excess, ovarian dysfunction and early loss of female fertility, resembling human polycystic ovary symptom (PCOS). Current studies suggest that PI3K signaling in TI cells plays a critical role in regulating the expression of *Lhcgr*, steroidogenic genes key for androgen biosynthesis, folliculogenesis during postnatal ovarian development.

2. Materials and methods

2.1. Animals and genotyping

Floxed *Pten* (Lesche et al., 2002) and *ROSA26R* (Soriano, 1999) mice were purchased from the Jackson Lab, Bar Harbor, ME). *Cyp17iCre* mice (Bridges et al., 2008) were imported from the University of Kentucky (Lexington, KY). $Pten^{fl/fl}$ mice were crossed with *Cyp17iCre* to generate $Pten^{fl/fl}$;*CypiCre* mice, similar to our previous reports (Lan et al., 2003a). Mouse tail genomic DNA was extracted using ZR genomic DNA-tissue mini prep kits according to the procedure recommended by the manufacturer (Zymo Research Corp, Irvine, CA) and genotyped by PCR using reported primer sets for $Pten^{fl}$, $Pten^{\Delta 5}$ (*Pten* exon 5 deletion allele), *R26R* allele and the *Cyp17iCre* transgene as listed in Table 1.

For *in vivo* treatment of AKT inhibitor API-2 (Bio-Techne, Minneapolis, MN), mice at postnatal day (PD) 30 were injected intraperitoneally (i.p.) daily with vehicle (1:9 v/v of DMSO/0.9% NaCl) or API-2 (1 mg/kg body weight) for 30 days (Yang et al., 2004). For *in vivo* luteinizing hormone (LH) treatment, mice at PD22 - 24 were i.p. injected daily with saline or 0.5 IU LH for 5 days. Human LH was obtained from the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA.

All animals were housed on a 14-h light:10-h dark cycle with food and water provided *ad libitum* and maintained in the pathogen-free vivarium. The studies have been approved by the Animal Care and Use Committee of the University of Louisville. All mice were sacrificed under ketamine anesthesia and all efforts were made to minimize their discomfort.

2.2. Histological and morphometric analysis of the ovaries

Ovaries were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and embedded in paraffin. Serial seven μm thick cross-sections were cut and used for hematoxylin and eosin (H & E) and immunohistochemistry staining. For histological analysis and follicle counting, serial sections at seven μm thickness were cut and stained with H & E. Every seventh section was assessed for the number of primordial, primary, secondary, preantral, antral and atretic follicles as well as corpora lutea using an Olympus light microscope and a modification of the Pedersen staging (Pedersen and Peters, 1968; Emmen et al., 2005; Hegele-Hartung et al., 2004). To verify *Cyp17iCre* expression in the ovaries, frozen sections of R26R:*Cyp17iCre* ovaries were stained for β-galactosidase using a kit according to the manufacturer's suggested procedure (Sigma, St Louis, MO).

2.3. Immunohistochemical staining

The procedure was performed by an avidin-biotin immunoperoxidase method as described previously (Lei et al., 2001). Briefly, deparaffinized sections were rehydrated and then incubated with 1% H_2O_2 for 30 min. After rinsing with PBS and incubated with normal serum at room temperature (RT), the sections were incubated with rabbit anti-Pten antibody (1:500, R & D Systems Inc, Minneapolis, MN) overnight at 4° C and then incubated with biotinylated secondary antibody (1:100, Vector Laboratories, Burlingame, CA) for 1 h at RT. After rinsing with PBS, sections were incubated with avidin-biotin-horseradish peroxidase complex using a Vectastain ABC kit (Vector Laboratories) for 1hr and rinsed with PBS. Immunostaining was detected by incubation of the sections with the substrate 3′3-diaminobenzidine at RT. All sections were counterstained with methyl green. Replacement of the primary antibody with irrelevant rabbit or mouse IgG was used as a procedure control.

2.4. Isolation of granulosa cells, TI cells, oocytes and cumulus cells

Ovaries isolated from PD60 mice with the bursa removed were placed in RPMI 1640 medium. Granulosa cells were collected by multiple follicle punctures using 30-gauge needles followed by filtration through 40-μm cell strainers (BD Falcon, Bedford, MA) to remove oocytes. TI cells were isolated as reported with minor modifications (Magoffin and Erickson, 1988; Li and Hearn, 2000). Briefly, ovaries from PD30 mice were punctured using 30-gauge needles to completely release antral granulosa/oocyte complexes. Punctured ovaries were then digested with 0.1% collagenase and 0.01% DNase I to release theca and interstitial cells. Tissue digestion was terminated when theca layers from most preantral follicles were released into the solution, while most preantral GC-oocyte complexes remained intact, similar to the method of isolation of preantral granulosa cells (Eppig and Telfer, 1993; Latham et al., 2004). TI cells were obtained by filtration of the enzyme-digested solutions through 40-μm cell strainers to remove preantral granulosa cells/oocyte complexes followed by centrifugation through a 44% Percoll gradient to remove blood cells (Magoffin and Erickson, 1988; Li and Hearn, 2000). Oocytes and cumulus cells were collected from one-month-old mice after PMSG/hCG treatment as described previously (Elvin et al., 2000).

2.5. RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA from ovary, TI and granulosa cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (Yuan et al., 2010). Briefly, total RNA was adjusted to a concentration of approximately 1.0 μg/μL. Two microgram total RNA were reverse transcribed into cDNA with random primers (Invitrogen) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp, Madison, WI). The cDNA was amplified by PCR with the primer sets of *Pten* and a housekeeping gene, β-actin (*Actb*). PCR primers as listed in Table 1 were designed according to the sequences obtained from GenBank using the Vector NTI 12.0 program (Invitrogen) and synthesized by Operon Technologies (Alameda, CA). All primers were designed to amplify the products that covered more than one exon. Each PCR-cycle consisted of denaturation for 45 s at 94 °C, annealing for 1 min at 57 °C, and extension for 1 min at 72 °C for 28 cycles. The amplified products were separated by electrophoresis in agarose gels and stained by