

SHORT COMMUNICATION

Immune-labeling of cytochrome P450scc and its associate electron-transferring enzymes in mouse ovarian tissue sections

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Abstract

Steroid hormone biosynthesis is mediated by cytochrome P450 side-chain cleavage (cytP450scc) enzyme, a rate-limiting step involved in the conversion of cholesterol to pregnenolone in gonads. This enzyme is accompanied by electron-transferring enzymes, such as adrenodoxin and adrenodoxin reductase, all localized to the inner mitochondrial membrane. Immunostaining was performed on mouse ovarian tissue sections at various stages of folliculogenesis using antisera generated against cytP450scc, adrenodoxin, and adrenodoxin reductase. Additional staining was conducted using the CF488A-conjugated phalloidin and CF555-conjugated wheat germ agglutinin lectin to identify different stages of folliculogenesis and ovulation cycle. Staining for lectin-binding moieties and phalloidin was observed in the zona pellucida region of early preantral and antral follicles. Expression of P450scc, adrenodoxin, and adrenodoxin reductase enzymes was detected in thecal cells of primordial, primary, and secondary follicles, as well as interstitial stromal cells. The findings presented here may contribute to identifying the specific roles of cytP450scc-expressing cells in the pathogenesis of polycystic ovarian syndrome and autoimmune primary ovarian insufficiency, including cases caused by autoimmune oophoritis.

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1. Background

In mice, the estrous cycle is divided into four stages, such as proestrus, estrus, metestrus, and diestrus, which repeat every 4 – 5 days unless there is interruption by pregnancy, pseudopregnancy, or anestrus.¹ Mouse ovary consists of ovarian follicle cells that convert into oocytes, and somatic cells differentiate into granulosa and theca cells. Several evidence indicate that steroidal and non-steroidal factors produced by granulosa and theca cells play an important part in the development and differentiation of both cell types during folliculogenesis.²

Cholesterol side-chain cleavage enzymes are encoded by gene cytochrome P450 Family 11 subfamily A member 1 (CYP11A1). The cytochrome P450scc (cytP450scc)

enzyme, localized in thecal, stromal, and interstitial cells of ovarian tissue, catalyzes the cleavage of cholesterol side-chain with the help of adrenodoxin reductase and adrenodoxin, which shuttle electrons to cytP450scc. Adrenodoxin reductase is a member of the flavoproteins reductase family, whereas adrenodoxin is a member of the iron-sulfur protein family.³⁻⁵ The subcellular localization studies using immunogold labeling have identified cytP450scc within the mitochondrial space of theca interna cells and corpus luteum cells.⁶ Similarly, in subcultured granulosa cells transformed with the Ha-ras oncogene (PO-GRS cell line), electron microscopy showed mitochondrial localization of cytP450scc, adrenodoxin, and adrenodoxin reductase enzymes.⁷ However, these techniques have significant limitations, including a lack of clear *in situ* visualization of these enzymes using specific probes.

In the current study, the cellular architecture of ovarian tissue was first mapped, followed by the examination of the *in situ* localization of mitochondrial cytP450scc, adrenodoxin, and adrenodoxin reductase enzymes using a high-resolution microscopy imaging technique.

2. Materials and methods

2.1 Antibodies and fluorescent tags

Antisera against adrenodoxin, adrenodoxin reductase, and cytP450scc were generated in the laboratory of Prof. Israel Hanukoglu.⁸ The specificities of these mitochondrial enzyme antibodies were confirmed using mouse testicular parenchyma, with representative images demonstrating the specificity of the adrenodoxin antibody shown in Figures 1 and 2. The secondary antibody, Alexa Fluor 555 goat anti-rabbit immunoglobulin G (IgG) (H+L) (A21428), was purchased from Life Technologies. The CF488A-conjugated phalloidin (BTM-00042) and CF555 conjugated-wheat germ agglutinin (WGA) (BTM-29076-1) were purchased from Biotium Inc. In addition, FITC-conjugated WGA (L4895-2MG) was purchased from Sigma-Aldrich.

2.2. Animals

A 3-month-old female Sabra mouse was housed according to the standard laboratory conditions with ad libitum access to food and water. The study protocol was approved by the Institutional Animal Ethics Committee of Ariel University (Ariel, Israel) (permit 32_12733_b019) in accordance with the Ministry of Health guidelines. Ovaries were surgically removed immediately after the CO₂ asphyxiation of each animal. The samples were kept overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM potassium phosphate, pH 7.4, and

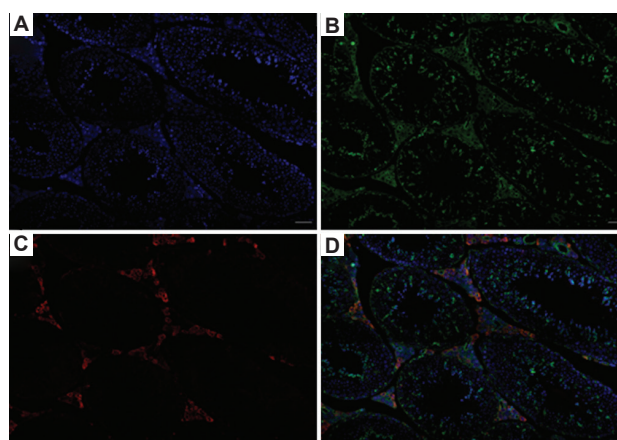


Figure 1. Immunofluorescence localization of adrenodoxin in mouse testicular parenchyma. (A) 4',6-diamidino-2-phenylindole staining highlights the cell nuclei. (B) Phalloidin staining shows actin filament localization in cross-sections of mouse testes, indicating different stages of sperm development. (C) Adrenodoxin staining is observed in Leydig cells located within the interstitial cells surrounding the seminiferous tubules. (D) Merged image of panels A, B, and C. Scale bar: 50 μ m.

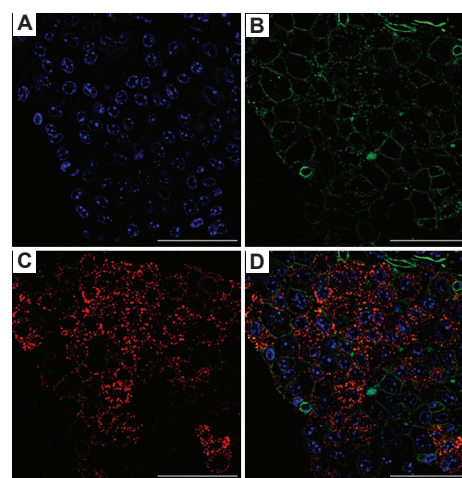


Figure 2. High-resolution image of adrenodoxin localization in Leydig cells within the interstitial cell population comprising fibroblasts, macrophage, and major steroid hormone-producing Leydig cells. (A) 4',6-diamidino-2-phenylindole staining for interstitial tissue present within the seminiferous tubules. (B) CF488A conjugated Phalloidin staining shows actin filaments within the interstitial tissues. (C) Mitochondrial enzyme ADX localized within the interstitial cells surrounded by seminiferous tubules. (D) Merged image of panels A, B, and C.

150 mM NaCl). Subsequently, samples were transferred to 30% sucrose and kept at 4°C for at least 24 h. The samples were then embedded in an optimal cutting temperature compound (Tissue-Tek, Sakura, Netherlands) and stored at -80°C until further use.

2.3. Cryotomy and immunofluorescence staining

The tissue blocks were sectioned at a thickness of 30 μ m using a cryostat (Leica Jung Frigocut 2000, Wetzlar,

Germany) at -25°C and collected in PBS containing 0.1% sodium azide. For immunofluorescence labeling, tissue sections were permeabilized with 0.1% Tween-20 (Sigma-Aldrich) in PBS for 10 min, followed by three washes in PBS for 5 min each. The sections were blocked in 300 μL of 4% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 20 min and incubated at room temperature for 1 h with different antisera against adrenodoxin, adrenodoxin reductase, or cytP450scc at a 1:50 dilution in PBS containing 2% BSA. Sections were then washed 6 times in PBS (5 min each) and incubated overnight at 4°C with the secondary antibody, goat anti-rabbit IgG (1:200 dilution), in PBS containing 2% BSA. After six additional PBS washes (5 min each), nuclei were stained with 4'-diamidino-2-phenylindole (DAPI) for 2 min.

Unless otherwise indicated, all the steps were carried out at room temperature. In control experiments, the same protocol was followed except for primary antiserum which was either omitted or replaced by normal rabbit serum. In these control slides, only DAPI nuclear staining was observed. To stain actin filaments, tissue sections were incubated with CF488A-conjugated phalloidin (1:20 dilution) in PBS for 45 min at room temperature. The lectin binding sites in the sections were stained with either FITC- or CF555-conjugated WGA (5:1000 dilution) in PBS for 30 – 45 min.

The sections were mounted onto X-tra Adhesive slides (Leica Biosystems, Peterborough, UK) using an anti-fade reagent glycerol containing n-propyl gallate (Sigma-Aldrich) in 100 mM phosphate buffer (pH 7.2). All experiments were performed at least 3 times, each yielding independent results.

2.4. Confocal microscopy

High-resolution fluorescent images were acquired using an LSM 700 confocal microscope (Carl-Zeiss, Germany). The laser diodes used for image acquisition were 405 nm for DAPI, 488 nm for CF488A, and 555 nm for Alexa Fluor 555 and DyLight™ 554. The fluorescence and bright-field illumination modes were used during the image acquisition process. Samples were visualized through LCI Plan-Apochromat $\times 25/0.8$, EC Plan-Neofluar $\times 40/1.30$, and Plan-Apochromat $\times 63/1.40$ oil-immersion objective lenses. The composite images were generated using the tile-scan image overview mode.

3. Results

The current study identified subcellular localization of cytP450scc, adrenodoxin, and adrenodoxin reductase enzymes in mouse ovarian tissue sections. The results are described in the sections below.

3.1. Actin and lectin localization

Actin filaments are fundamental structural components of the cytoskeleton, formed by the polymerization of actin moieties that regulate cellular functions such as cell migration, cell adhesion, and the formation of cell protrusions. Actin polymerization adapts different conformational changes, forming structures that range from densely packed lamellipodia to large contractile bundles in mesenchymal cells.^{9,10} Actin filaments are also known to play vital roles in oocyte maturation, with the role of filamentous actin (F-actin) confirmed in chromosome segregation during oocyte meiosis.¹¹ Moreover, the actin network has been linked to long-range vesicle transport, nuclear repositioning, spindle migration, and anchorage in mammalian oocytes.¹²

Thirty-micrometer-thick tissue sections were stained with CF555-conjugated WGA and CF488A-conjugated phalloidin. Phalloidin, a class of toxins belonging to phallotoxins found on death-cap mushrooms, binds specifically to filamentous actin, enabling visualization of actin filaments across the ovarian sections (Figure 3). Staining for lectin-binding moieties and phalloidin was observed in the zona pellucida region of early preantral and antral follicles (Figure 3A and B). However, no lectin-binding signal was detected in late atretic follicles (Figure 3A). Phalloidin staining, along with DAPI nuclear staining, clearly distinguished different phases of ovarian follicle development (Figure 3B-D). The images show various stages of follicular development, including primordial and primary follicles, early preantral and antral follicles, and late atretic follicles.

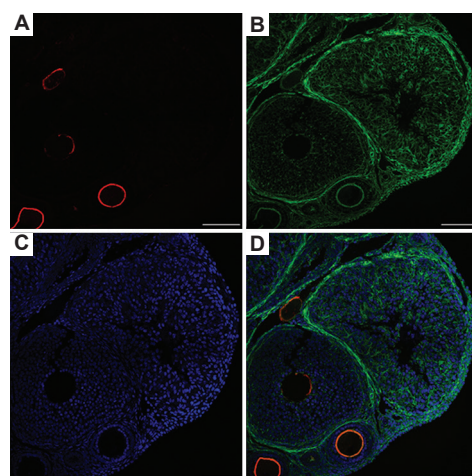


Figure 3. Representative immunofluorescence staining of a mouse ovarian cross-section. (A) Lectin-binding moieties are localized to the zona pellucida regions of ovarian follicles. (B) CF488A-conjugated phalloidin staining shows actin filaments in the mouse ovarian cross-section. (C) 4'-diamidino-2-phenylindole staining highlights the cell nuclei. (D) Merged image of panels A, B, and C. Scale bar: 50 μm .

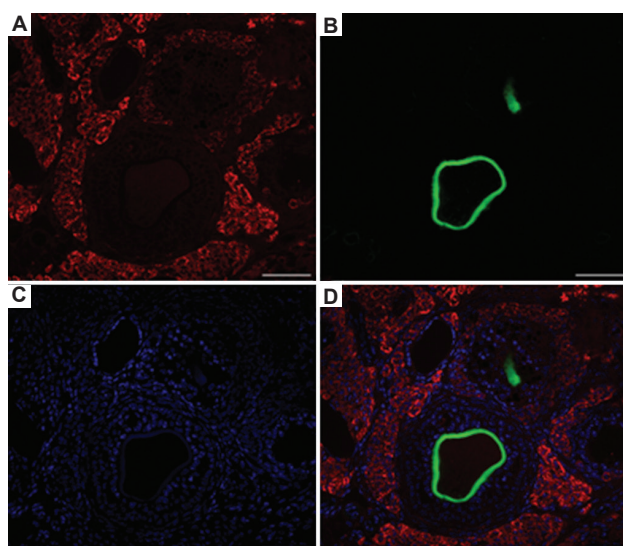


Figure 4. Immunofluorescence localization of cytP450scc in mouse ovarian follicles, shown alongside lectin and nuclear staining. (A) Red fluorescence shows the specific localization of cytP450scc enzymes within the thecal cells and interstitial stromal cells. (B) Lectin staining is localized to the zona pellucida region of the secondary follicle. (C) 4',6-diamidino-2-phenylindole staining highlights the cell nuclei. (D) Merged image of panels A, B, and C. Scale bar: 50 μ m.

Abbreviation: cytP450scc: Cytochrome P450 side-chain cleavage.

3.2. CytP450scc

Several enzymes from the P450 family are expressed in mouse gonads. Some, such as Cyp11a and Cyp17a1, are involved in the biosynthesis of steroid hormones, while others are involved in metabolizing the xenobiotic compounds.^{13,14} Three cytP450 proteins, Cyp2b19, 3a57, and 4f39, have been found to be highly expressed in mouse testicular parenchyma, whereas Cyp11b1 is predominantly expressed in ovaries.^{15,16} The CYP11A1 gene, a member of the cytP450 family 11, catalyzes a crucial step in the conversion of cholesterol to pregnenolone in developing avian granulosa cells.^{17,18}

In this study, mouse ovarian tissue sections were stained with cytP450scc antibodies along with phalloidin (Figure 4A). Strong phalloidin staining was observed in the zona pellucida region of primary follicles (Figure 4B). CytP450scc expression was detected in thecal cells of primordial, primary, and secondary follicles, as well as in interstitial stromal cells. However, no expression was observed in granulosa cells within the mouse ovarian cross-sections (Figure 4C and D). In contrast, cytP450scc expression was present in both follicular thecal and follicular granulosa cells of the corpora lutea (Figure 5A-C). The corpora lutea, which form once the ovum is released from follicles, serves as a major source of the hormone progesterone.¹⁹

3.3. Adrenodoxin reductase and adrenodoxin

Adrenodoxin reductase is a monomeric 51 kDa flavoenzyme expressed in all steroidogenic tissues and serves as the first electron transfer protein in the mitochondrial cytP450 system, including the cholesterol side-chain cleavage pathway. Adrenodoxin reductase receives a two-electron package from nicotinamide adenine dinucleotide phosphate (NADPH) and converts it into two single electrons through adrenodoxin to mitochondrial cytP450.²⁰

In the current study, 25 μ m-thick mouse ovarian sections were stained with adrenodoxin reductase and adrenodoxin antibodies. Immunoreactivity for adrenodoxin reductase and adrenodoxin proteins was observed in the thecal cells and interstitial stromal cells lining the follicles (Figures 6A-C and 7A-C).

These staining patterns were consistent with those of cytP450scc staining in mouse ovarian tissue sections. However, no staining was detected in granulosa cells of follicles at any developmental stage. To cross-check the specificity of these steroidogenic mitochondrial enzymes, mouse testicular parenchyma was stained with adrenodoxin antibodies at different stages of sperm maturation.

Adrenodoxin staining was present only in interstitial cells of mouse testicular sections (Figure 1C and D). The interstitial compartment of the testicular parenchyma comprised various cell types, including Leydig cells, immune cells, and fibroblasts.²¹ High-resolution imaging of testicular parenchyma stained for adrenodoxin resulted in specific localization within Leydig cells (Figure 2C and D). These results confirm the expression of cytP450scc, adrenodoxin reductase, and adrenodoxin, all parts of the steroidogenic mitochondrial system, in gonadal cells involved in steroid hormone biosynthesis.

4. Discussion

CytP450scc is a classical cholesterol side-chain cleavage enzyme that catalyzes the removal of six carbons from the side chain of cholesterol and converts into pregnenolone, the first rate-limiting step in mammalian steroid hormone biosynthesis^{22,23} along with adrenodoxin and adrenodoxin reductase. These mitochondrial enzymes are on the mitochondrial membrane, with adrenodoxin predominantly localized to the inner mitochondrial membrane, as evident by an electron microscope.⁷ Adrenodoxin, which transfers electrons to cytP450scc, has been widely studied and is highly essential for steroid hormone biosynthesis or steroidogenesis.²⁴

Ovaries secrete multiple steroid hormones, such as pregnenolone, progesterone, 17 α -hydroxyprogesterone,

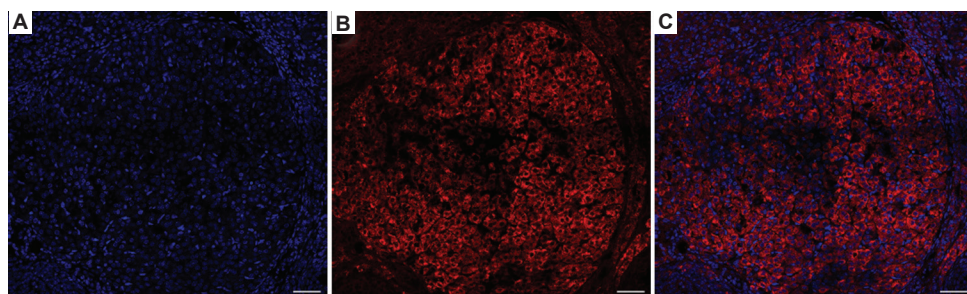


Figure 5. Immunofluorescence localization of cytP450scc in mouse corpora lutea. (A) CytP450scc localization is observed in follicular granulosa cells. (B) 4',6'-diamidino-2-phenylindole staining highlights the cell nuclei. (C) Merged image of panels A and B. Scale bar: 50 μ m. Abbreviation: cytP450scc: Cytochrome P450 side-chain cleavage.

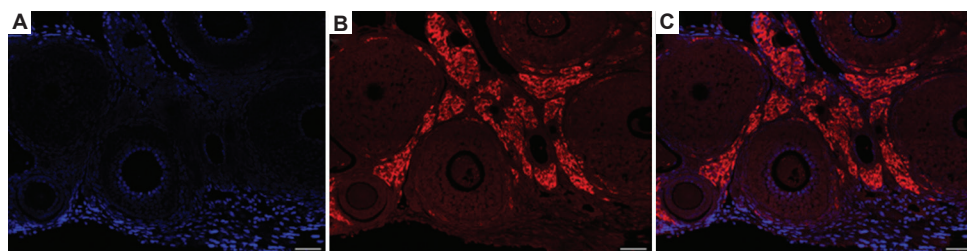


Figure 6. Immunofluorescence localization of adrenodoxin reductase in mouse ovarian cross-section. (A) 4',6'-diamidino-2-phenylindole staining highlights the cell nuclei. (B) Adrenodoxin reductase staining is mostly observed in the thecal cells and interstitial cells. (C) Merged image of panels A and B. Scale bar: 50 μ m.

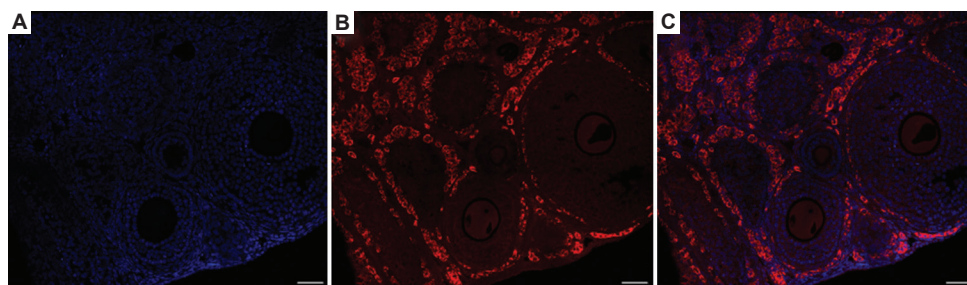


Figure 7. Immunofluorescence localization of adrenodoxin in mouse ovarian cross-section. (A) 4',6'-diamidino-2-phenylindole staining highlights the cell nuclei. (B) Adrenodoxin staining is mostly observed in the thecal cells of mouse ovarian follicles and interstitial stromal cells. (C) Merged image of panels A and B. Scale bar: 50 μ m.

androstenedione, testosterone, estrone, and estradiol, whose levels fluctuate depending on the estrous cycle. While thecal and follicular granulosa cells are the primary somatic cell types responsible for steroid hormone production, interstitial stromal cells also stained positive for cytP450scc enzyme (Figures 4D, 6C, and 7C) and have already been shown to produce steroids in epithelial ovarian tumor cells.²⁵ The human genome contains a total of 57 P450 genes categorized into two classes, which are type I and type II. Type I enzymes, localized on the mitochondrial membrane, receive electrons from NADPH mediated by ferredoxin and ferredoxin reductase. Type II enzymes are located on the endoplasmic reticulum and receive electrons from NADPH mediated by P450 oxidoreductase. The cytP450scc expression is required for steroid hormone

production but is also considered an antigen associated with polycystic ovarian syndrome and autoimmune primary ovarian insufficiency, including cases caused by autoimmune oophoritis.^{26,27} Hence, the current study may serve as a valuable resource for elucidating the role of the cytP450 system in disease pathogenesis and several other regulatory aspects of steroid hormone biosynthesis.

5. Conclusion

The current study evaluates the localization of cytP450scc, adrenodoxin, and adrenodoxin reductase, key rate-limiting enzymes involved in the biosynthesis of steroid hormones, in the mammalian gonadal system. Results clearly indicate that identifying cell types in mouse ovarian tissues that express these enzymes would be greatly beneficial in

understanding the pathogenesis of autoimmune disorders affecting steroid-producing cells, such as autoimmune oophoritis.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: Sachin Sharma

Investigation: Sachin Sharma

Methodology: Sachin Sharma

Visualization: Sachin Sharma

Writing – original draft: All authors

Writing – review & editing: Kalimuthu Kalishwaralal

Ethics approval and consent to participate

For animal use in research conducted for this study, ethical approval was sought from the Institutional Animal Ethics Committee of Ariel University (Ariel, Israel) (permit 32_12733_b019) in accordance with Ministry of Health guidelines. This study did not involve human participants as subjects and employed the study design of human-related clinical trials in this research.

Consent for publication

Not applicable.

Availability of data

Microscopy data related to this study are available from the corresponding author upon request.

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