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RESEARCH ARTICLE

# Characterization of Mitochondrial Heat Shock Protein 60 variants in HEK293 Cells Transformed into Steroidogenic

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ABSTRACT

**Introduction:** During pregnancy, P4 is essential to maintain the maternal-fetal relationship. Maternal cholesterol is the main source of P4 production, a process that takes place in the syncytiotrophoblast mitochondrion. The mechanism and proteins involved in the cholesterol transport for the steroidogenic process are still unknown in detail. The STARD3 protein could be the substitute for its STARD1 equivalent localized in all acute response tissues. However, mutation or null STARD3 mice maintain their reproductive capacity, suggesting other proteins are involved in this process. Previously, we reported that the HSP60 participates in steroidogenesis in mitochondria isolated from the placental syncytiotrophoblast, mitochondrial contact sites or JEG-3.

Also, take relevance that non-steroidogenic cells, such as the HEK293, which are human kidney embryo cells, when are transformed into steroidogenic by transfection of the steroidogenic machinery, they synthesize progesterone.

To understand better the mechanism through which HSP60 participates in placental steroidogenesis, mutation of cysteine 442, which is essential in the active site for its activity, and deletion of 146 amino acid residues of the N-terminal of HSP60 were performed. The first was implemented to determine whether the protein structure is essential to support steroidogenesis, and the second was done to elucidate whether its activity occurs outside or inside the mitochondrion.

**Methods:** Two mutants were obtained: a) cysteine 442 was replaced by alanine ($HSP60^{C442A}$) and b) the HSP60-mature ($HSP60^{M}$) without the mitochondrial-leading sequence. Human kidney cells HEK293 were transformed into steroidogenic by transfection with pECE-P450scc, pCMV-3βHSD-I. The transfected cells were transfected with the $HSP60^{wt}$, $HSP60^{C442A}$, or $HSP60^{M}$ plasmids. The transfection was validated by western blot and P4 was determined by an enzyme immunoassay kit. HSP60 without mutations was used as control ($HSP60^{wt}$).

**Results:** The synthesis of P4 was stimulated by the wild type HSP60 ($HSP60^{wt}$). However, with both mutants, steroidogenesis occurred as in the control, suggesting that mutants do not support P4 synthesis.

**Discussion:** The mechanism to transport cholesterol to steroidogenic mitochondria requires the full HSP60 to support P4 synthesis, which is necessary to maintain pregnancy.

**Highlights**

- HSP60 participates in the steroidogenesis of transformed HEK293 cells.
- $Cys^{442}$ mutant of HSP60 loses its activity in steroidogenesis.
- N-terminal deletion of HSP60 is not involved in steroidogenesis.
- Native HSP60 is critical for steroidogenesis.

**Keywords**: Steroidogenesis, transformed HEK293, HSP60, P4 synthesis.

## Introduction

The human placenta plays a key role during pregnancy, allowing for its successful coming to term. It anchors the fetus to the uterus, allows the exchange of nutrients and waste between the maternal and fetal circulation, and has immunomodulatory properties[1-4].

One of the main hormones synthesized by the placenta is progesterone (P4), which is exclusively supplied by the placenta from the 6th to 7th week of pregnancy when luteal production declines[5]. P4 is key to maintain the maternal-fetal relationship[6] because it promotes blastocyst implantation, extracellular remodeling, and trophoblast migration since early pregnancy, it protects the fetus and promotes its development; therefore, low levels of P4 end in abortions or preterm-delivery, confirming its key role during pregnancy[7-9].

P4 is synthesized in syncytiotrophoblast mitochondria from cholesterol derived from maternal low-density lipoproteins (LDLs), which are incorporated into the cell by endosomes. Although the steroidogenic acute regulatory protein 1 (STARD1) is the principal protein associated with the cholesterol flux in mitochondria from acute response steroidogenic tissues, it is not expressed in the human placenta[10], even though high P4 levels are needed during pregnancy[11].

Cholesterol for P4 synthesis in the placenta derives not only from LDLs but also from high-density lipoproteins (HDLs) via receptors. Once LDLs and HDLs are in the cell via receptor-mediated endocytosis, the lipoproteins are transported to lysosomes via endocytic compartments, where cholesterol is released, and cholesterol esters are hydrolyzed by the lysosomal acid lipase[12-14].

Also, a member of the STARD family, STARD3 (previously MNL64, metastatic lymph node 64) has been described in breast and ovarian carcinomas[15]. This protein shares 37% of amino acid similarity with STARD1[16,17] which has been identified in the syncytiotrophoblast with a molecular weight of 50 kDa and has been suggested to play the role of STARD1 in the human placenta[18]. STARD3 is mainly located in late endosomes, where it co-localizes with proteins, Niemann–Pick type C2 (NPC2) and C1 (NPC1), facilitating the transport of cholesterol to the plasma membrane[19].

Late endosomes anchor to the outer mitochondrial membrane (OMM) through the soluble NSF attachment protein-receptor (SNARE) complex, where STARD3 undergoes proteolytic activation into a 24-kDa protein[20] and associates with the mitochondrial contact sites, facilitating cholesterol flow from OMM to the inner mitochondrial membrane (IMM)[21]. Alternatively, endosomes in combination with the endoplasmic reticulum form the mitochondria-associated membranes[22-24] or the STARD3-VAP (vesicle-associated membrane protein [VAMP]-associated proteins [VAPs]) complex[25].

However, the role of STARD3 has been doubted because a targeted mutation to the STARD domain has no significant effects on steroidogenesis[26]. On the other hand, when STARD3 was reduced in 80-90% by siRNA, P4 synthesis decreased only 30%, which suggested that there must be other proteins involved in placental steroidogenesis[26].

Until today, there is no knowledge on how cholesterol is transported into mitochondria to be transformed in P4. Even more, in the human placenta, the supply of cholesterol to syncytiotrophoblast mitochondria must be ensured, as high concentrations of P4 are required to maintain pregnancy. If the 80-90% by siRNA reduction of STARD3 affects only 30% of P4 synthesis, then other proteins could participate in cholesterol transport in syncytiotrophoblast cells to produce the required P4.

In this sense, looking for the mechanism of cholesterol transport in mitochondria from the syncytiotrophoblast, we identified a mitochondrial heat shock protein 60 (HSP60) that shared an 18.6% of identity with STARD3[27]. It has been described that HSP60 has a $Cys^{442}$ that is central to its activity[28]. Because of the reactivity of Cys with maleimides, the HSP60 function was inhibited with fluorescein-5-maleimide, resulting in a significant decrease of P4 synthesis in mitochondria isolated from the syncytiotrophoblast, JEG-3 cells, and mitochondrial contact sites[27]. On the other hand, the overexpression of HSP60 in JEG-3 cells increases the synthesis of P4[29]. In the same sense, the addition of recombinant His10-HSP60 to mitochondria isolated from JEG-3 cells increases P4 synthesis in a dose-dependent manner, as well as in non-steroidogenic HEK293 cells transfected with the electron transport chain coupled to P450scc and the 3β-hydroxy steroid dehydrogenase type I enzyme (3β-HSD-I)[29]. These results suggest that HSP60 participates in the mitochondrial steroidogenesis of the syncytiotrophoblast.

The question arises whether the mitochondrial participation of HSP60 in steroidogenesis is required as a native protein structure and whether HPS60 acts inside or outside mitochondria. Therefore, the objective of this work was to further understand the role of HSP60 in placental

steroidogenesis. To reach this objective, two mutants were implemented. The first mutant exchanges the amino acid $Cys^{442}$ from the active site of HSP60 with alanine ($HSP60^{C442A}$) to determine if the HSP60 requires its mature conformation. The second corresponds to the HSP60-mature ($HSP60^{M}$) without the mitochondrial leading sequence (aa's 1-123) and was used to determine if its activity is required inside or outside mitochondria. The model used corresponded to the non-steroidogenic human kidney HEK293 cells that were transformed into steroidogenic by the transfection with the electron transport chain coupled to the P450scc and the 3β-HSD-I enzyme. The HSP60 mutants were transfected to these cells. The results support that the HSP60 participates in steroidogenesis.

## Materials and Methods

PLASMIDS AND STRAINS

The plasmid pGEM-HSP60 encoding human mitochondrial HSP60 was provided by Dr. Radhey S. Gupta, McMaster University, Canada. pCMV-3βHSD-I (encoding 3β-hydroxysteroid dehydrogenase/$\Delta^5$-$\Delta^4$-isomerase type I) and pECE-P450scc (for human cytochrome P450scc system, P450scc-AdRed-Adx) plasmids were provided by Dr. Jerome Strauss III, Virginia University, USA.

Competent *Escherichia coli* TOP10 cells were transformed with 1 to 50 ng of circular DNA and incubated in an ice bath for 60 min, followed by 60 sec of heat shock at 42°C. After the incubation, cells were returned to the ice for 60 min at 4°C in SOB medium (LB medium plus 25 mM KCl and 10 mM $MgCl_2$, pH 7.0). The transformed cells were cultures on LB agar plates with 100 µg/mL ampicillin and incubated at 37°C overnight. Isolated colonies were selected to inoculate 5 mL of LB-ampicillin liquid medium at 37°C overnight. Plasmids were purified using the ZR Plasmid Miniprep kit (Zymo Research Co., Irvine, CA, USA) and sequenced at the *Instituto de Fisiología Celular, UNAM*, Mexico.

WILD TYPE HSP60

Overexpression of the HSP60 protein was achieved by producing a mammalian expression vector. The construct pGEM-HSP60 was sub-cloned into the expression plasmid pECE and named HSP60 wild type ($HSP60^{WT}$). The reaction was carried out performing a PCR reaction with the FW primer for the complete HSP60: 5´- GCGGTACCATGCTTC GGTTACCC- 3´ and the RV primer HSP60 5´-CGGAGCTCTTAGAACATGCCACCTCC- 3´; *KpnI* and *SacI* restriction sites were introduced at the 5' and 3' ends (underlined) in the FW and RV primers, respectively. PCR products were obtained using a thermocycler (Axygen® MaxyGene II, Corning Inc., Corning, NY, USA) ). Subsequently, the amplified fragment and the pECE vector were digested with *KpnI* and *SacI* enzymes (New England BioLabs, Ipswich, MA, USA).

AMPLIFICATION OF MATURE HSP60

The sequence corresponding to the mature HSP60 protein ($HSP60^{M}$, nucleotide 78-1722) was amplified using $HSP60^{wt}$ as template, the FW primer 5'- GCGGTACCATGGCCAAAGATGTAAAAT-3' and the previous reverse primer. This step eliminated the first 26 amino acids, which correspond to the leader sequence (Fig. Supp.1). Afterwards, the 1640 bp product was sub-cloned into the empty pECE vector with the same restriction sites (Fig. 1).

SITE-DIRECTED MUTAGENESIS FOR $HSP60^{C442A}$

$HSP60^{wt}$ gene was used as template for mutagenesis. Linearized DNA fragments were produced with mutagenic ends and a point mutation. Two primers were used to amplify the full-length human HSP60 for site-directed mutagenesis of C442A 22. (Fig. Supp. 2)[30].

The following FW primer 5'-GGGAGGGGGTGCGGCCCTCCTTC-3' and RV primer 5'- GAAGGAGGGCCGCACCCCTCCC-3´ were used to substitute cysteine (triplet TGT) for an alanine (triplet GCG). Mutations were performed in three-step PCR[31].

In the first PCR, the forward flank (*KpnI*) primer and reverse mutagenic primer (MR) were used. For the second PCR, the forward mutagenic primer (MF) and the reverse flank (SacI) primer were used. PCR reaction mixtures contained 1X buffer, 50 ng DNA, 0.5 µM primers, 200 µM dNTP mixture, 1.5 mM $MgCl_2$, and 1U Phusion DNA polymerase (New England BioLabs). Finally, the desired mutation was generated in a third PCR by using the previous PCR products as templates (200 ng each) and the forward (*KpnI*) and reverse (*SacI*) primers (Fig. 2A-B).

All PCR conditions were carried out using the following program: first cycle of denaturation at 95°C for 5 min, and 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 5 min, and a final extension at 72°C for 5 min. The resulting fragments from the three PCRs were visualized on 1% agarose gel and purified using Zymoclean™ Gel DNA Recovery kit (Zymo Research Co.).

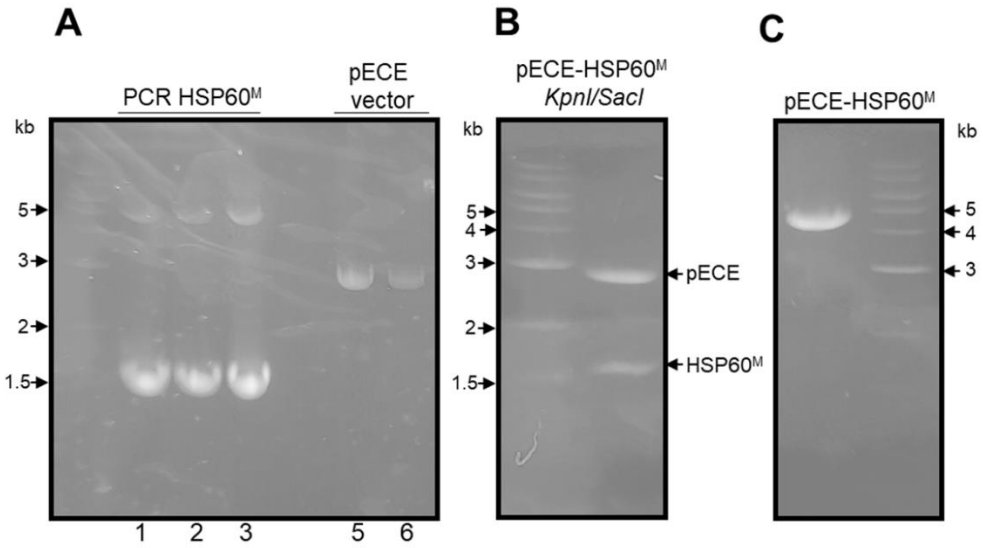


**Figure 1**. *Amplification of the mature HSP60*. **A**) PCR product of HSP60$^{M}$ (1640 bp) (line 1-3) using HSP60$^{wt}$ as template, and empty pECE linear vector (2910 pb) (line 5-6). **B**) The digestion of pECE-HSP60$^{M}$ plasmid with *KpnI* and *SacI r*estriction enzymes, showing two bands corresponding to the pECE plasmid and the mature HSP60 (nucleotide 78-1722). **C**) Linearized pECE-HSP60$^{M}$ with *KpnI* enzyme.

The HSP60$^{C442A}$ mature protein was sub-cloned in the pECE vector replacing the complete HSP60$^{wt}$ protein. Both, vector and HSP60$^{C442A}$, were digested with *EcoRI* and *SacI* enzymes. Digestions were analyzed in a 1% agarose gel electrophoresis and the fragments of interest: a 3668-bp fragment (which consisted of a 2910-bp pECE vector and a 758-pb HSP60$^{wt}$ initial fragment) and a 1010-bp HSP60$^{C442A}$ fragment were cut from the gel and purified (Fig. Supp. 1). Ligation of the purified fragments was performed using a 1:3 proportion (New England Biolabs) (Fig. 2C).

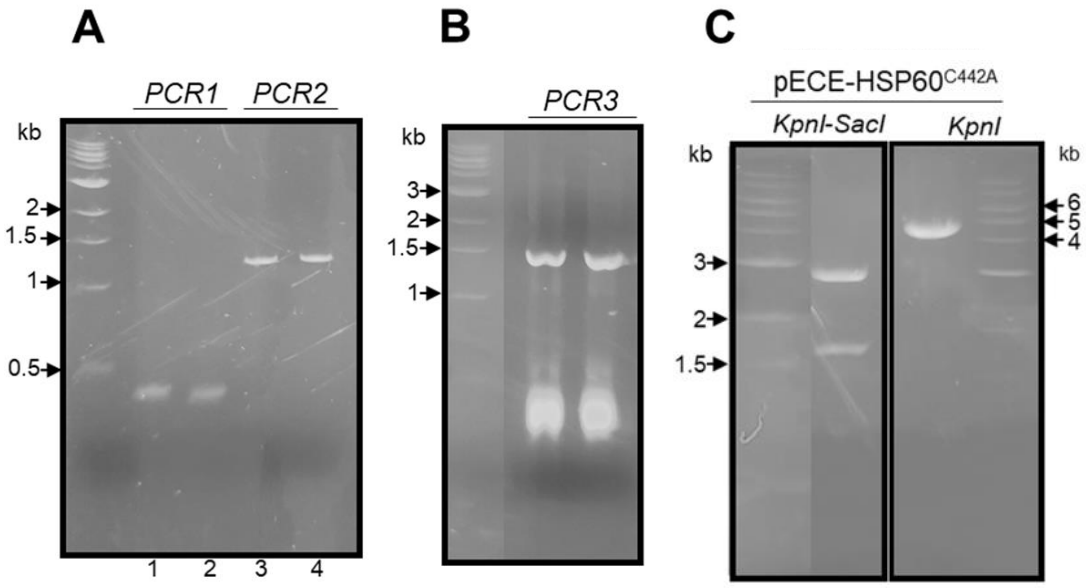


**Figure 2**. *Site-directed mutagenesis for HSP60*$^{C442A}$. Mutations were performed in three-step PCR using HSP60$^{wt}$ as template. **A**) PCR1, with forward flank (*KpnI*) primer and reverse mutagenic primer (line 1-2); PCR2, forward mutagenic primer and reverse flank (*SacI*) primer (line 3-4). **B**) PCR3 product was attained using PCR1 and PCR2 as template with *KpnI* and *SacI* restriction sites primers. **C**) pECE-HSP60$^{C442A}$ was digested with *KpnI* and *SacI r*estriction enzymes. Two bands were obtained, pECE vector (2910 bp) and HSP60$^{C442A}$ (1722 bp). Entire construct linearized with *KpnI* with expected band size (4632 bp).

CELL CULTURE AND TRANSIENT TRANSFECTION

Human kidney cells HEK293 (ATCC, VA, USA) were plated at a culture density of 1 × $10^5$ viable cells in 24-well plates in DMEM medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 20 mM HEPES, pH 7.4. Cells were grown at 37°C under 95% air, 5% $CO_2$ in a humidified atmosphere. Cells were transiently co-transfected with the FuGENE®6 reagent (Promega, Madison, WI, USA) and 250 ng of pECE-P450scc, pCMV-3βHSD-I plus $HSP60^{wt}$, $HSP60^{C442A}$, or $HSP60^{M}$ plasmids. After 24 h of transfection, 100 μg/mL of LDL was added as a source of cholesterol.

P4 DETERMINATION

P4 synthesis was quantified at 72 h post-transfection using a P4 Enzyme Immunoassay Kit (EIA-1561, DRG Diagnostics GmbH, Marburg, HE, Germany), according to the manufacturer's protocol.

WESTERN BLOTTING

Cells were scraped and incubated in lysis buffer (50 mM NaCl, 5 mM EDTA, 5 mM Tris–HCl, 1% Igepal™, and protease inhibitors) for total protein extraction. Protein (30 μg/line) was loaded onto 12% SDS-PAGE[32] and western blot was performed as described elsewhere[33]. PVDF (Millipore, Darmstadt, Germany) membranes were incubated overnight with antihuman HSP60-COOH (1:8000; sc-13966), 3β-HSD-1 (1:300; sc-515120), actin (1:2000; sc-1616) and P450scc (CYP11A1; 1:500; sc-292456) from Santa Cruz Biotechnology, Paso Robles, CA, USA. The protein-antibody complexes were detected using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). The protein concentration was determined by the Bradford method[34] using albumin as standard.

STATISTICAL ANALYSIS

Data were analyzed in GraphPad Prism version 9. One-way ANOVA analysis and a post hoc test of two-stage linear step-up were performed to compare means and control the false discovery rate (FDR)[35].

## Results

Co-transfection of HEK293 cells with P450scc and 3βHSD-I was supported by western blot after 72 h of treatment (Fig. 3). Also, the effectiveness of the transfection of these cells with $HSP60^{wt}$, $HSP60^{M}$, or $HSP60^{C442A}$ was confirmed by western blot (Fig. 3). The co-transfection of non-steroidogenic HEK293 cells resulted in cells being transformed into steroidogenic (Fig. 4). It is important to mention that although P450scc and 3βHSD-I were identified in non-transfected HEK293 cells, P4 synthesis was not stimulated. Reports exist on 3βHSD-II-IV isoforms expressed in kidney tissue[36,37] and a low expression of 3β-HSD-I in the HEK293 cell line[38]. Similarly, there is a report on the P450scc (CYP11A1) protein expressed in adult rat kidneys, with the ability to synthesize pregnenolone, although at deficient levels[39]. These reports could explain the signal detection of these proteins by the antibodies in the controls.

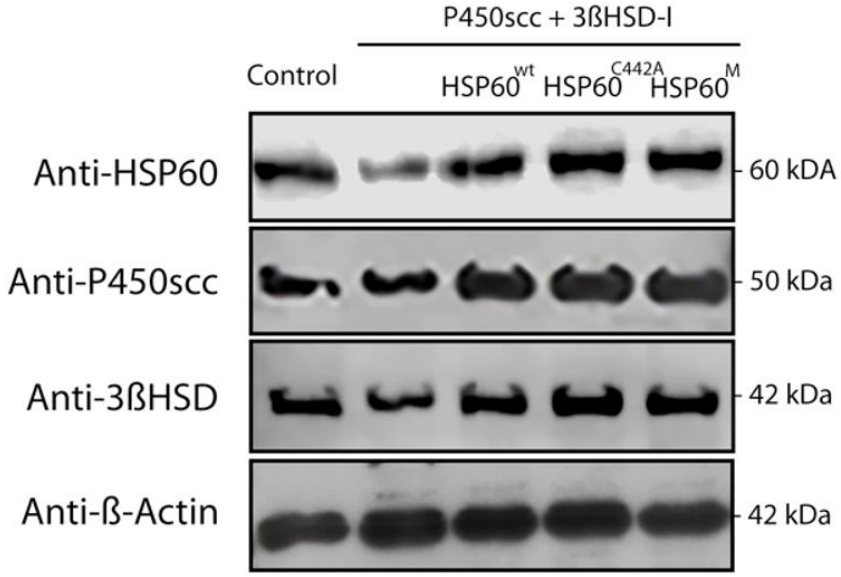


**Figure 3**. *Transfection efficiency by western blot analysis*. After 72 h of treatment, protein expression was determined via western blotting. HEK293 cells were transfected with pECE-P450scc and PCMV-3BHSD-I plus $HSP60^{wt}$, $HSP60^{C442A}$ or $HSP60^{M}$. Representative western blot of five independent experiments.

As reported previously[29], we found that overexpression of HSP60$^{wt}$ significantly increased P4 production in HEK293 cells co-transfected with 3β-HSD-I and P450scc, compared to cells transfected only with 3β-HSD-I and P450scc (Fig. 4). In contrast, the overexpression of the HSP60$^{C442A}$ mutant decreased the P4 synthesis even below those transfected only with 3β-HSD-I and P450scc. The mature HSP60 peptide also produced P4 levels equivalent to those transfected only with 3β-HSD-I plus P450scc and above the levels of the HSP60$^{C442A}$ mutant.

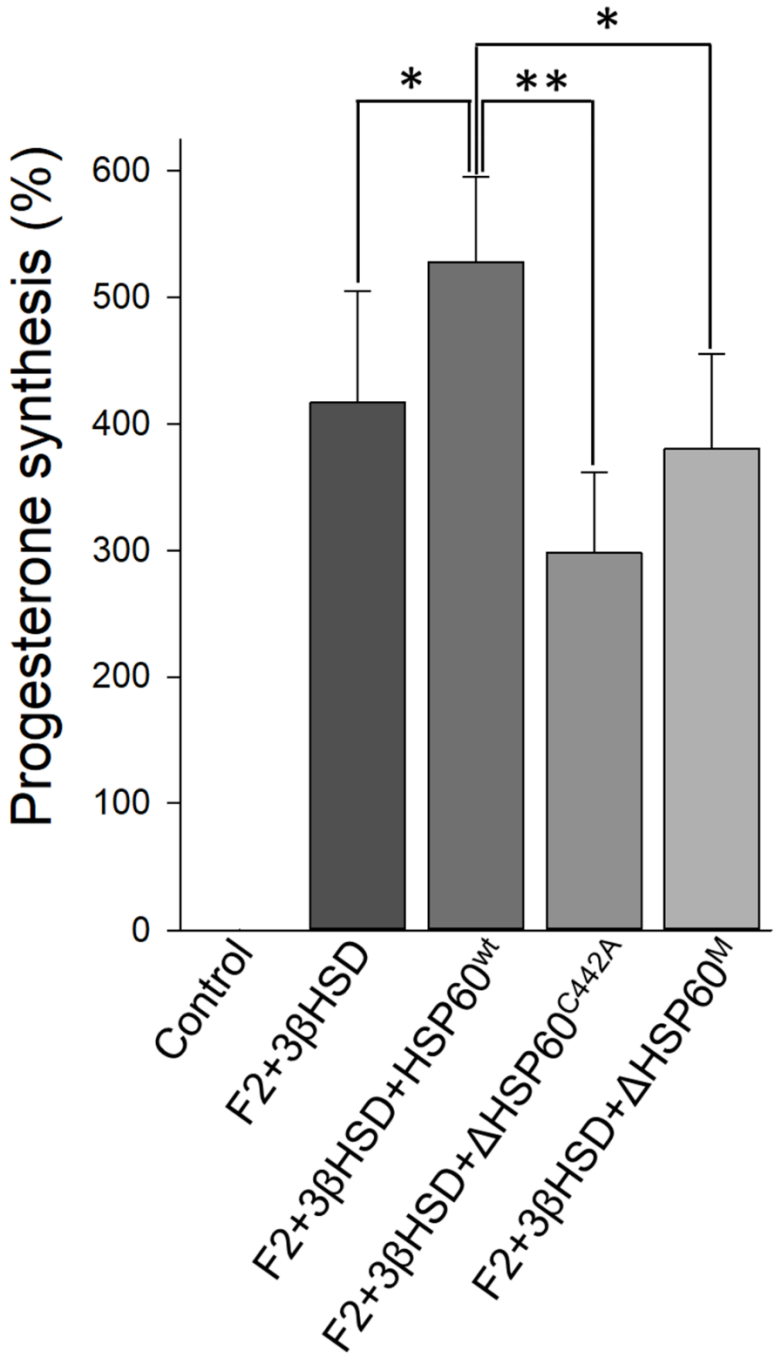


**Figure 4**. *HSP60$^{C442A}$ decreased P4 synthesis in transformed HEK293 cells*. HEK293 cells were co-transfected with pECE-HSP60$^{C442A}$ or HSP60$^{M}$ as described under Methods. After 72 h, P4 was quantified in the culture medium. P4 concentration is expressed as percentage of the control value (mean ± SD, *p < 0.01 and **p < 0.001 mean statistical difference assessed by ANOVA analysis and two-stage linear step-up of test, as reported in [27]; n= 5).

## Discussion

The human HSP60 has a $Cys^{442}$ located near the ATP-binding site, which plays an important role in its chaperone activity and oligomer formation[40]. The transcriptional modifications, like S-guanylation in $Cys^{442}$, can disturb the multichaperone complex stability and, therefore, alter the opening of the mitochondrial permeability pore[41]. In addition, covalent binding of metabolites like epolactaene or ETB (epolactaene tertiary butyl ester) to $Cys^{442}$ can induce the inhibition of HSP60 activity by disrupting oligomerization[28] or inducing conformational changes of the apical domain, responsible for the recognition of the co-chaperone HSP10[42]. In this sense, N-ethyl-maleimide or fluorescein-5-maleimide modified the activity of HSP60 in steroidogenic cells by blocking cysteines through a thiol-maleimide reaction[27] that inhibits the protein activity by blocking cysteines[43].

In the present study, the non-steroidogenic HEK293 cells were used to have a system where the steroidogenesis is not part of the cells, hence, the effect of HSP60 could reflect the participation of this protein in the synthesis of steroids. In this sense, the HEK293 cells were transfected to overexpress the P450scc and 3βHSD-I proteins. As mentioned, there are reports that describe the presence of 3βHSD-II-IV isoforms in kidney tissue[36,37], as well as low expression of 3β-HSD-I in the HEK293 cell line[38]. Even more, the presence of P450scc (CYP11A) expressed in adult rat kidneys has also been reported[39], with the ability to synthesize pregnenolone because HEK293 synthesizes HSP60. This could explain the low P4 synthesis under control conditions and the identification of these proteins by western blot.

The effect of HSP60 on the steroidogenesis in transformed cells was confirmed, the P4 concentration in the culture medium increased when $HSP60^{wt}$ was overexpressed (Fig. 4). It is important to mention that the 20% increase between control and the HSP60-transfected HEK293 cells is lower than in the previous report[27]. This is because the plasmids used in this study were modified to transfect the HEK283 cells with the mutants, and this could affect the efficiency of transfection. Nevertheless, HSP60, under these experimental conditions, increased P4 synthesis.

Furthermore, the modification in the $Cys^{442}$ residue to generate the $HSP60^{C442A}$ mutant diminished P4 synthesis compared to the wild type HSP60. In the same way, the $HSP60^{M}$ mutant did not support P4 synthesis, because it needs its mitochondrial location, as has been proposed in syncytiotrophoblast mitochondria[27, 29].

Since modification of the $Cys^{442}$ residue interferes with HSP60 oligomerization and can affect protein-protein interactions as occurs with the HSP10, there is a high possibility that HSP60's role in steroidogenesis might be related to its folding function. It has been proven to participate in other roles beside protein folding[44] and because modification of $Cys^{442}$ affects the adjacent binding pocket[28], it is likely that such modification also affects HSP60's interaction with other molecules, such as the apoptotic role exerted by HSP60 in tumor cells via formation of a multichaperone complex, as mentioned above[41]. The finding that HSP60 interacts with STARD3 and P450scc[29] supports the hypothesis that HSP60's role in steroidogenesis is associated with the mitochondrion.

Recently, it was described that HSP60 regulates P450scc accumulation remodeling the mitochondrial crista[45]. The changes in mitochondrial structure have been associated with an increase in placental steroidogenesis[46], which could be related to ATP synthesis[47]. Interestingly, HSP60 could take different oligomeric conformations[48,49] expressing several functions inside mitochondria, even in the cytosol, or extracellularly depending on the HSP60's association with other proteins or post-translational modifications[50].

## Conclusions

Our findings that mitochondrial HSP60 is involved in steroidogenesis are supported by the results shown in this paper. Furthermore, the use of cells transformed into steroidogenic enable to test the role of HSP60 on the steroidogenic process in a specific way. Overexpression of $HSP60^{M}$ and $HSP60^{C442A}$ variants clearly shows that protein $HSP60^{wt}$ has a central role in the synthesis of progesterone. However, further studies are needed to determine the exact role HSP60 plays in P4 synthesis in the human placenta.

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## Disclosure of interests

All authors declare they have no conflict of interest.

## Ethical approval for studies involving humans

This article does not contain any studies with human participants performed by any of the authors.

## Ethical approval for studies involving animals

This article does not contain any studies with animals performed by any of the authors.

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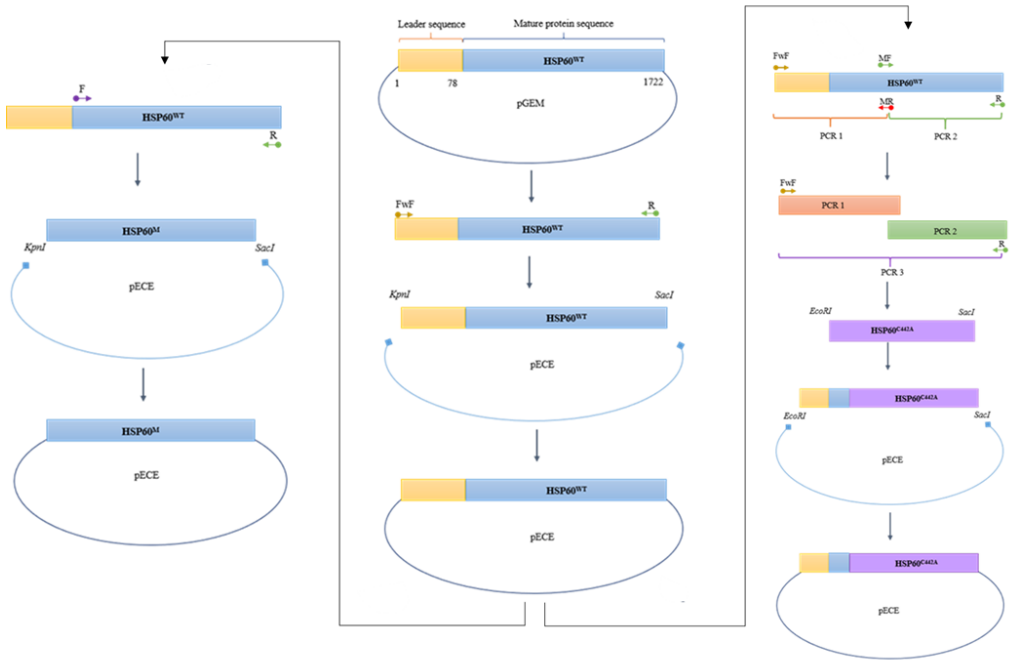
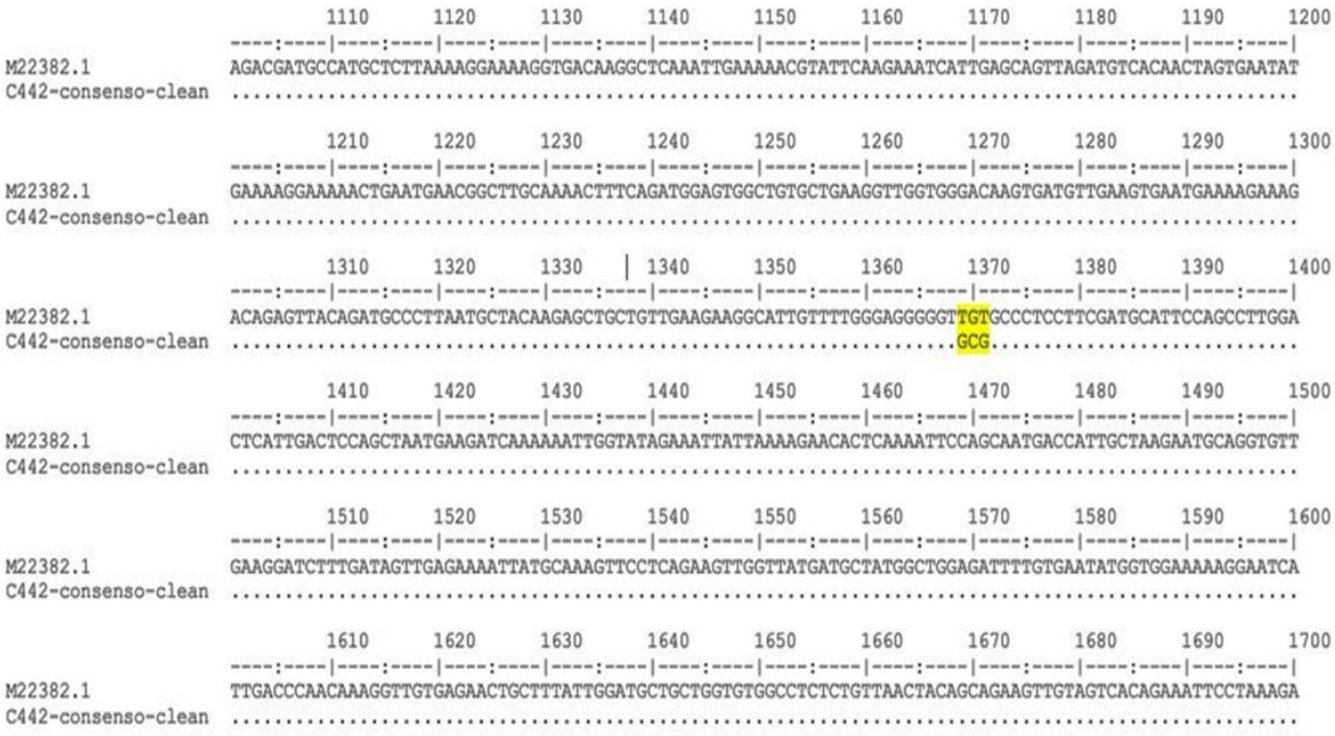
## SUPPLEMENTARY MATERIAL




**Supplementary Figure 1**. Schematic representation of the mature protein and the C442A mutant construction by site-directed mutagenesis. FwF: Forward primer complete; F: Forward primer; R: Reverse primer; MF: mutant forward primer; MR: mutant reverse primer.




**Supplementary Figure 2.** Part of the alignment of the human HSP60 signaling codon 442 replacing the amino acid Cys with Ala.