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Isocitrate dehydrogenase type 2 (IDH2) is part of a multiprotein complex for placental steroidogenesis

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ABSTRACT

Background: Human syncytiotrophoblast mitochondria require the activity of the isocitrate dehydrogenase type 2 (IDH2) to obtain reduced coenzymes for progesterone (P4) synthesis. Data from the literature indicate that mitochondrial steroidogenic contact sites transform efficiently cholesterol into P4. In this research, we identified the IDH2 as a member of the steroidogenic contact site and analyzed the steroidogenic role of its activity.

Method: Human syncytiotrophoblast mitochondria were isolated by differential centrifugation, and steroidogenic contact sites were obtained by osmotic shock and sucrose gradient ultracentrifugation. In-gel native activity assay, mass spectroscopy, and western blot were used to identify the association of proteins and their activities. P4 was determined by immunofluorescence.

Results: The IDH2 was mainly identified in steroidogenic contact sites, and its activity was associated with a complex of proteins with an apparent molecular mass of ~590 kDa. Mass spectroscopy showed many groups of proteins with several metabolic functions, including steroidogenesis and ATP synthesis. The IDH2 activity was coupled to P4 synthesis since in the presence of Ca^{2+} or Na_2SeO_3, inhibitors of the IDH2, the P4 production decreased.

Conclusions: The human syncytiotrophoblast mitochondria build contact sites for steroidogenesis. The IDH2, a non-membrane protein, supplies the NADPH required for the synthesis of P4 in a complex (steroidosome) that associate the proteins required to transform efficiently cholesterol into P4, which is necessary in pregnancy to maintain the relationship between mother and fetus.

General significance: The IDH2 is proposed as a check point in the regulation of placental steroidogenesis.

1. Introduction

Progesterone (P4) is essential to maintain the relationship between mother and fetus, since its decrease produces abortion [1]. The synthesis of P4 in the first weeks of pregnancy occurs in the corpus luteum, but the placenta is the tissue responsible for P4 production during the remainder of pregnancy. Human syncytiotrophoblast mitochondria (HSM) are responsible for transforming cholesterol into P4 [2,3].

The cholesterol used by syncytiotrophoblast cells for P4 synthesis is obtained from the maternal LDL, which is captured by endocytosis. Once inside the cells, cholesterol is transported to mitochondria by a process mediated by specific proteins [4,5].

From the outer mitochondrial membrane (OMM), cholesterol is translocated to the inner mitochondrial membrane (IMM) to be transformed in pregnenolone (P5) by cytochrome P450scc (P450scc) [2,3]. The isocitrate dehydrogenase type 2 (IDH2), a soluble enzyme from the mitochondrial matrix, provides the electrons as NADPH to P450scc through adrenodoxin reductase (AdxR) and adrenodoxin (Adx) [6–8]. Thereafter, P5 is transformed into P4 by the 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase type 1 (3β-HSD1) activity, which is located in the inner mitochondrial membrane [9,10].

Cholesterol transport has been associated to mitochondrial contact sites, where several proteins guarantee its delivery to the cytochrome P450scc [11,12]. A mitochondrial contact site is a close approximation

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Abbreviations

P4	progesterone
P450scc	cytochrome P450 side chain cleavage
IDH2	isocitrate dehydrogenase type 2
IDH3	isocitrate dehydrogenase type 3
SN	supernatant
HCM	human cytotrophoblast mitochondria
HSM1	crude human syncytiotrophoblast mitochondria
HSM	purified human syncytiotrophoblast mitochondria
OMM	outer mitochondrial membrane
IMM	inner mitochondrial membrane
SF	soluble fraction corresponding to the intermembrane space and matrix of mitochondria
SCS	steroidogenic contact site
hrCN-PAGE	high resolution clear native-PAGE
AdxR	adrenodoxin reductase
Adx	adrenodoxin
3β-HSD1	3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase type 1
HSP60	heat shock protein 60
ANT	adenine nucleotide translocator
VDAC	voltage-dependent anion channel

between the outer and inner membranes where specific proteins enable the exchange of molecules between the cytoplasm and mitochondria. These structures are dynamic and participate in modulating cellular metabolism [13].

Previously, we described the isolation of steroidogenic contact sites (SCS) from enriched inner mitochondrial membrane of syncytiotrophoblasts; these contact sites synthetized P4 in a medium containing isocitrate and $NADP^+$ [12]. This observation suggested that all the necessary elements for the synthesis of P4 should be in this fraction [14]. Thereafter, it was shown that the activity of the IDH2 in HSM was inhibited in the presence of 1 μM Ca^{2+}, and this inhibition was associated with a 65% reduction of P4 synthesis [15].

Although IDH2 participation seems to be essential for the synthesis of P4, there is no information about its relationship with other steroidogenic proteins in HSM. To get a deeper insight, we determined the association of the IDH2 with SCS by using hrCN-PAGE and MS/MS analyses. Also, western blot and immunoprecipitation with several antibodies against the electron transport chain coupled to P450scc were performed. Finally, P4 synthesis was evaluated in the IMM and SCS in the presence of Ca^{2+} or sodium selenite (Na_2SeO_3), which are inhibitors of IDH2 activity. The results showed that SCS have the IDH2 associated tightly with the P450scc electron transport chain conforming a multiprotein complex.

2. Material and methods

2.1. Reagents

All chemical reagents were analytical grade. All the antibodies were purchased from Santa Cruz Biotechnology®, Inc. (USA). IDH2 (sc-374476), P450scc (sc-292456), HSP60 (sc-13966), ANT (sc-11433), VDAC (sc-8829), 3β-HSD1 (sc-28206), AdxR (sc-374436). Total OXPHOS Human WB Antibody Cocktail (ab110411) was purchased from Abcam®, it contains mitochondrial antibodies against: complex I subunit NDUFB8, complex II subunit 30 kDa, complex III subunit Core 2, complex IV subunit II, and ATP synthase subunit alpha.

2.2. Isolation of human syncytiotrophoblast mitochondria

This protocol was approved by the Human Research Ethics Committee from the *Instituto Nacional de Ciencias Médicas y Nutrición, "Salvador Zubirán"* (No. BRE-2510-18/21–1) and the Internal Review Board of the *Hospital General, "Dr. Manuel Gea González"* in Mexico City (No. 11-57-2016). Written informed consent was obtained from all participants. All pregnant women were from an urban area of Mexico City, 18–39-year-old, previously normotensive, with no history of diabetes mellitus or thyroid, liver, renal disease. Term placentae (39–41 weeks of gestation) were acquired from uncomplicated pregnancies; twin pregnancies were excluded from this study. Placentas were collected immediately following normal delivery and HSM were isolated –as previously reported [16]. The whole procedure was performed at 4 °C. The cotyledons were cut superficially avoiding connective tissue and placed in a solution containing 250 mM sucrose, 1 mM EDTA, pH 7.4 (sucrose-EDTA). The tissue was homogenized twice using a Polytron PT10 at 3000 rpm for 45 s with a 1-min interval. The pH of the homogenate was adjusted to 7.4 with Tris-base, and centrifuged at 1380 *g* for 15 min. The supernatant was centrifuged at 3836 *g* for 10 min. The pellet was discarded and the supernatant containing the crude human syncytiotrophoblast mitochondria (HSM1) was recovered and centrifuged at 18,566 *g* for 15 min. The pellet was diluted in 30 mL of sucrose-EDTA, and centrifuged at 11,872 *g* for 15 min at 4 °C. To separate any remaining erythrocytes, the pellet was placed in a solution containing 35% sucrose (δ 1.151 g/mL), 10 mM Tris-base, pH 7.4, and centrifuged at 27,712 *g* for 45 min, obtaining the purified HSM (Supplemental Fig. 1). The pellet was diluted with 30 mL of sucrose-EDTA, and centrifuged at 17,096 *g* for 15 min. The HSM were resuspended in 0.5 mL of sucrose-EDTA supplemented with protease inhibitors and stored at −70 °C until use.

2.3. Isolation of submitochondrial fractions and steroidogenic contact sites (SCS)

The submitochondrial fractions OMM, IMM, and SCS were obtained following the method described by Uribe et al. [12]. Briefly, 25 mg of HSM protein obtained from at least three different placentas was incubated in an ice bath with 10 mM H_3PO_4, pH 7.3, and the Complete protease inhibitor cocktail for 20 min. After incubation, sucrose was added to attain a concentration of 0.382 M sucrose for 20 min and centrifuged at 12,500 *g* for 10 min. The supernatant, corresponding to OMM, was recovered and centrifuged at 137,000 *g*. Mitoplasts were incubated in 1 mM H_3PO_4 for 20 min and, then, 0.31 M sucrose was added. After incubation, IMM was recovered by centrifugation at 102,000 *g*. The IMM and OMM were washed with 0.25 M sucrose, 1 mM EDTA, pH 7.4. The soluble fraction (SF) containing the matrix and intermembrane proteins was concentrated by using an Amicon Stirred Ultrafiltration Cell of 3 kDa. Submitochondrial fractions were used immediately or kept at −70 °C until they were used.

The supernatant that contained the OMM fraction was recovered. The pellet (IMM) was subjected to a second hypoosmotic and hyperosmotic shock. Both fractions, IMM and OMM, were centrifuged at 142,000 *g* for 1 h at 4 °C and the pellet was collected.

To obtain the contacts sites, the IMM sample was separated in a continuous sucrose gradient (15%–60%) at 96,000 *g* for 20 h. Fraction three (1.21 δ), containing the steroidogenic fraction (SCS), was used for IDH2 activity experiments (Supplemental Fig. 1). The protein concentration was determined using BSA as standard, as described [17].

2.4. Solubilization of mitochondrial membrane proteins

The HSM were solubilized with digitonin at a 2:1 ratio (g detergent/g protein) as described [18], with some modifications. The HSM were incubated for 30 min at 4 °C in a solution containing 50 mM Bis-tris, 0.5 M aminocaproic acid, 10 mM succinate, 10 mM ATP, pH 7, under constant stirring [19]. Digitonin was added by dripping it to the sample

until reaching the required ratio. After incubation, the solution was centrifuged at 100,000 *g* for 35 min at 4 °C. The supernatant was recovered for the analysis of the protein complexes.

2.5. Electrophoresis in native conditions

The protein complexes (100 μg) were resolved under native conditions in a linear polyacrylamide gradient gel (4%–10%) (hrCN-PAGE) [20]. The anode buffer contained 25 mM imidazole, pH 7; the cathode buffer contained 50 mM tricine, 7.5 mM imidazole, pH 7, 0.05% DOC and 0.01% n-dodecyl β-D-maltoside (DDM) [21]. The Ponceau red dye was used as a run-ahead marker. Complexes of digitonin-solubilized bovine heart mitochondria were used as molecular weight standard.

2.6. In-gel activity of the isocitrate dehydrogenase type 2 (IDH2)

Upon completion of the native electrophoresis, the gels were incubated in a solution containing 16 mM isocitrate, 2.3 μM $MgCl_2$, 40 mM Tris-base, 1 mM $NADP^+$, 0.2 μM phenazine methosulfate (PMS), and 2 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The reaction was carried out in an orbital shaker in the dark at room temperature. The IDH2 activity was observed by the reduction of MTT, generating a purple precipitate. IDH3 was determined in the presence of 1 mM NAD^+ and, when it was present, 2 mM Ca^{2+} was added. The reaction was stopped with a solution of 40% acetic acid and 7% methanol.

2.7. Tandem mass spectrometry (LC/ESI-MS/MS)

For mass spectrometry, a band of ~590 kDa from SCS or HSM resolved under native conditions (hrCN-PAGE) was sent to the Analytical and Biological Mass Spectrometry Core Facility of the University of Arizona for proteomic analysis.

Proteins were analyzed by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS), which was performed by the Analytical and Biological Mass Spectrometry Core Facility at the University of Arizona. All samples submitted to the Mass Spectrometry were prepared following the specifications of the Mass Spectrometry Core Facility. At least three different samples were analyzed. Scaffold (version Scaffold_4.8.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from goat_human.gaf (downloaded Mar 14, 2019).

2.8. Progesterone synthesis

IMM or SCS (50 μg) were incubated 20 min at 37 °C in the following medium: 10 mM MES, 1 mM EGTA, 10 mM isocitrate, 120 mM KCl, 2 mM H_3PO_4, 2 mM ADP, pH 7.4, in the presence or absence of Ca^{2+} or Na_2SeO_3. The incubation medium to determine the transformation of cholesterol into P4 in the presence of Ca^{2+} was supplemented with 5 mM EGTA and 8 nM of free $CaCl_2$ by using the Chelator software to calculate the free Ca^{2+}. The 22-hydroxy cholesterol (22-OH-C) (10 μM) was used as control. The reaction was stopped in a cold-bath for at least 5 min and immediately centrifuged at 14,000 *g* during 10 min at 4 °C. The supernatant was collected and P4 quantified with a DRG Progesterone Enzyme Immunoassay Kit (EIA-1561 DRG Instruments GmbH, Germany) according to the manufacturer's protocol. All the samples were assayed in triplicate in at last three different experiments.

2.9. Immunoprecipitation

For immunoprecipitation (IP) of proteins, an affinity chromatography matrix was used. The procedure was carried out following the Protein G-Agarose (Roche®) manufacturer's instructions. SCS (1 mg) were treated with 50 μL of Protein G-Agarose (Roche®) and 0.8 μg of P450ssc, AdxR or IDH2 antibodies. The immunogenic complex was resuspended in 80 μL of loading buffer (0.02% bromophenol blue, 0.125 M Tris-HCl, 5% SDS, 25% glycerol, 5% β-mercaptoethanol) and heated to 100 °C for 5 min. The proteins were resolved in SDS-PAGE.

2.10. Western blot analysis

Proteins of HSM, IMM, OMM, SF, or SCS (25 μg each one) were

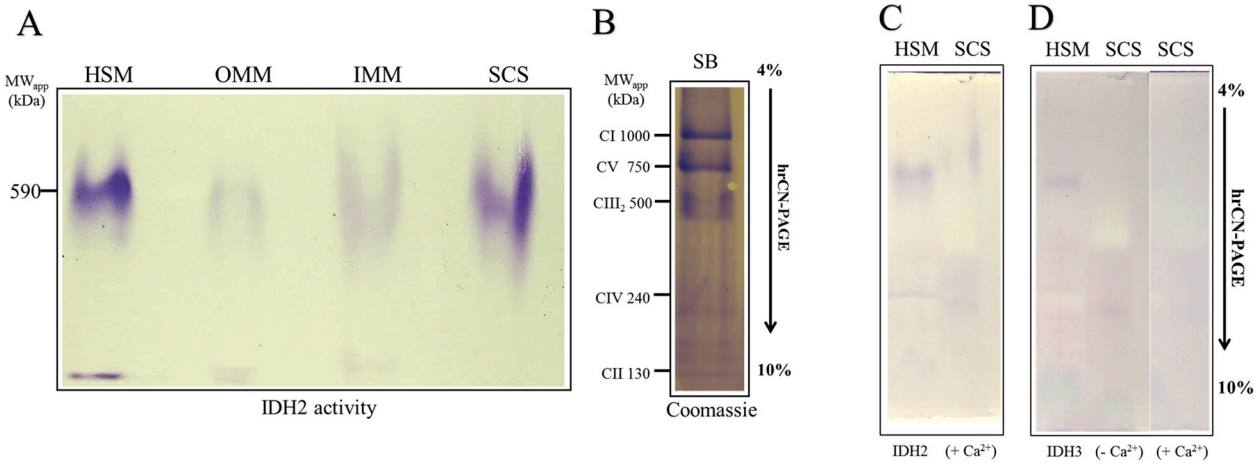


Fig. 1. In-gel activity of IDH2. **A**) HSM, IMM, OMM, and SCS proteins were solubilized with digitonin (2:1, g/g) and resolved in hrCN-PAGE. The purple (gray) precipitates represent the activity of the IDH2. **B**) Estimated molecular weight of the IDH2 activity spot by using solubilized bovine (SB) heart mitochondrial complexes with digitonin. **C**) HSM and SCS proteins were solubilized with digitonin (2:1, g/g) and resolved in hrCN-PAGE, representing the IDH2 activity in the presence of Ca^{2+}. **D**) The activity of IDH3 (IDH-NAD^+) was identified under similar experimental conditions. HSM: Human Syncytiotrophoblast Mitochondria; IMM: inner mitochondrial membrane; OMM: outer mitochondrial membrane; SF: soluble fraction; SCS: steroidogenic contact site. The samples were loaded with 100 mg of solubilized protein. The figure is a representative experiment of four independent determinations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Proteins associated to the 590-SCS isolated from HSM.

Steroidogenic proteins	Accession Number	MW kDa
Isocitrate dehydrogenase [NADP]	P48735/IDHP	51
Cholesterol side-chain cleavage enzyme	P05108/CP11A	60
3 beta-hydroxysteroid dehydrogenase/Delta 5– > 4-isomerase type 1	P14060/3BHS1	42
Aromatase	P11511/CP19A	58
NAD(P) transhydrogenase	Q13423/NNTM	114
Contact sites proteins		
Creatine kinase U-type	P12532/KCRU	47
Hexokinase-1	P19367/HXK1	102
Voltage-dependent anion-selective channel protein 2	P45880/VDAC2	32
60 heat shock protein	P10809/CH60	61
Carrier proteins		
Phosphate carrier protein	Q00325/MPCP	40
Mitochondrial carrier homolog 1	Q9NZJ7/MTCH1	42
Tricarboxylate transport protein	P53007/TXTP	34
Mitochondrial carrier homolog 2	Q9Y6C9/MTCH2	33
2-oxoglutarate/malate carrier protein	Q02978/M2OM	34
Calcium-binding carrier protein Aralar2	Q9UJS0/CMC2	74
Calcium-binding carrier protein Aralar1	O75746/CMC1	75
Calcium-binding carrier protein SCaMC-1	Q6NUK1/SCMC1	53
Krebs cycle proteins		
Pyruvate carboxylase	P11498/PYC	130
Pyruvate dehydrogenase E1 component subunit alpha	P08559/ODPA	43
Dihydrolipoyl dehydrogenase	P09622/DLDH	54
2-oxoglutarate dehydrogenase	Q02218/ODO1	116
Pyruvate dehydrogenase E1 component subunit beta	P11177/ODPB	39
Fumarate hydratase	P07954/FUMH	55
Isocitrate dehydrogenase [NAD] subunit gamma	P51553/IDH3G	43
Isocitrate dehydrogenase [NAD] subunit alpha	P50213/IDH3A	40
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	P31040/SDHA	73
Aconitate hydratase	Q99798/ACON	85
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	P21912/SDHB	32
Malate dehydrogenase	P40926/MDHM	36
Isocitrate dehydrogenase [NAD] subunit beta	O43837/IDH3B	42
Electron transport chains proteins		
Cytochrome b-c1 complex subunit 2	P22695/QCR2	48
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	O95299/NDUAA	41
Cytochrome b-c1 complex subunit 1	P31930/QCR1	53
Electron transfer flavoprotein-ubiquinone oxidoreductase	Q16134/ETFD	68
Complex I assembly factor TIMMDC1	Q9NPL8/TIDC1	32
NADH-cytochrome b5 reductase 3	P00387/NB5R3	34
Electron transfer flavoprotein subunit alpha	P13804/ETFA	35
Cytochrome c oxidase subunit 4 isoform 1	P13073/COX41	20
NADH-cytochrome b5 reductase 1	Q9UHQ9/NB5R1	34
Electron transfer flavoprotein subunit beta	P38117/ETFB	28
ATP synthase proteins		
ATP synthase subunit beta	P06576/ATPB	57
ATP synthase subunit alpha	P25705/ATPA	60
Heat shock proteins		
Stress-70 protein	P38646/GRP75	74
DnaJ homolog subfamily B member 11	Q9UBS4/DJB11	41
78 glucose-regulated protein	P11021/GRP78	72
10 heat shock protein	P61604/CH10	11
Metabolism of lipid proteins		
Carnitine O-acetyltransferase	P43155/CACP	71
Peroxisomal acyl-coenzyme A oxidase 3	O15254/ACOX3	78
Phosphatidylserine decarboxylase proenzyme	Q9UG56/PISD	47
Acyl-coenzyme A thioesterase 1	Q86TX2/ACOT1	46
Glycerol-3-phosphate dehydrogenase	P43304/GPDM	81
Carnitine O-palmitoyltransferase 2	P23786/CPT2	74
Very long-chain specific acyl-CoA dehydrogenase	P49748/ACADV	70

Proteins were analyzed by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS), which was performed by the Analytical and Biological Mass Spectrometry Core Facility at the University of Arizona. Three different samples were analyzed. Scaffold (version Scaffold_4.8.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications.

resolved in 12% polyacrylamide gels (SDS-PAGE) [22]. The proteins were electrotransferred to PVDF membranes in a semi-dry electroblotting system (Bio-Rad) [23] and processed for western blot analysis. The PVDF membranes were incubated overnight with primary antibody in Tween-TBS (0.05% Tween, 20 mM Tris-Base, 0.5 M NaCl, pH 7.5, and 5% defatted milk) in an orbital shaker at 4 °C. The membranes were washed to remove the unbound antibody with Tween-TBS and incubated with the corresponding secondary antibody. To detect the protein-antibody complex, the membrane was incubated with a chemiluminescent substrate coupled to peroxidase according to manufacturer's instructions (Immobilon Western HRP, Millipore). The loaded protein was evaluated by transillumination with 20% methanol before blotting [24].

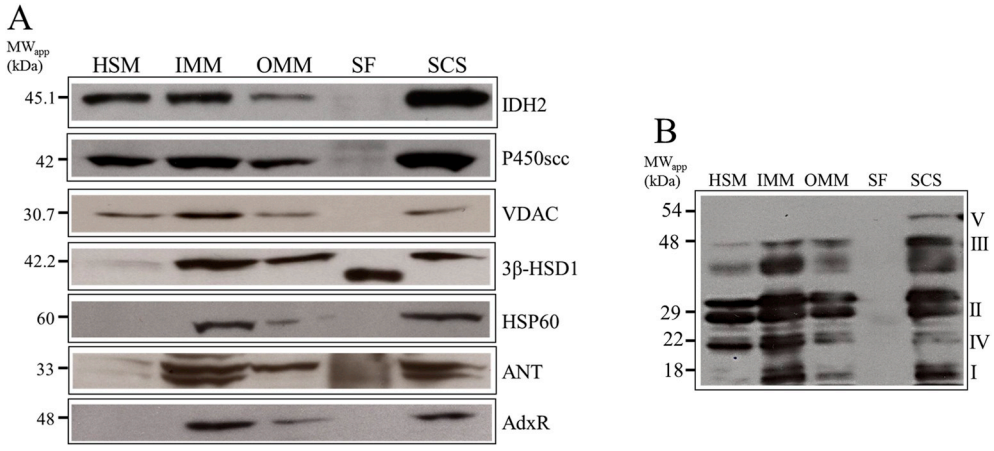


Fig. 2. Identification of proteins by western blot. **A**) Immunodetection of proteins associated with steroidogenesis in HSM, IMM, OMM, SF, and SCS: IDH2, P450scc, VDAC, 3β-HSD1, HSP60, ANT, AdxR. **B**) Immunodetection of proteins associated with oxidative phosphorylation (OXPHOS) complexes in HSM and submitochondrial fractions: CI subunit NDUFB8, CII SDHB, CIII UQCRC2, CIV MTCO1, and CV ATP5A. HSM: human syncytiotrophoblast mitochondria; IMM: inner mitochondrial membrane; OMM: outer mitochondrial membrane; SF: soluble fraction; SCS: steroidogenic contact site. MW, molecular weight. Protein load was 25 μg per lane. These are representative experiments performed in triplicate from independent samples.

2.11. Statistical analysis

A Student's t-test was used to statistical analysis with a statistical significance of $p \leq 0.05$ using the SigmaPlot software v.14.

3. Results

3.1. Identification of IDH2 activity in native conditions

The activity of the IDH2 was determined through hrCN-PAGE to establish its possible associations with other mitochondrial proteins. The treatment with digitonin showed that in the hrCN-PAGE, both HSM and SCS have IDH2 activity identified as a purple (gray) spot (Fig. 1A). The band corresponds to a molecular weight of ~590 kDa as compared to the mitochondrial bovine complexes (Fig. 1B). In comparison, the IMM and OMM showed a lower IDH2 activity (Fig. 1A).

It has been described that IDH2 activity in placental mitochondria is inhibited by Ca^{2+} [15]. To confirm that the activity observed was from the IDH2, the hrCN-PAGE gels with the HSM and SCS samples were incubated in the presence of 2 mM Ca^{2+}. Under these experimental conditions the IDH2 activity was inhibited significantly (Fig. 1C).

The activity of the mitochondrial NAD^+-dependent isocitrate dehydrogenase (IDH3) was also determined; however, in this case the activity of the enzyme was practically absent from the samples in the presence or absence of Ca2+ (Fig. 1D).

Since the molecular weight reported for the IDH2 is 50.9 kDa, the activity observed at ~590 kDa in the hrCN-PAGE strongly suggests that IDH2 could be part of a multiprotein complex.

3.2. Identity of the IDH2 partners in the protein band of ~590 kDa

To determine the proteins associated with IDH2, the band of ~590 kDa from SCS and HSM obtained from the hrCN-PAGE, referred as 590-SCS and 590-HSM, respectively, were sent to the Analytical and Biological Mass Spectrometry Core Facility from the University of Arizona, USA. From 321 proteins identified in 590-HSM and 590-SCS, 142 were shared between the 590-SCS and 590-HSM, whereas 84 were exclusively found in the 590-SCS and 95 in the 590-HSM.

Since the contact sites were previously described as steroidogenic, the first analysis performed to the MS results was the identification of the proteins associated with the transformation of cholesterol into P4. As observed in Table 1, the following proteins of 590-SCS associated with steroidogenesis were identified: P450scc, AdxR, 3β-HSD1, aromatase, $NAD(P)^+$ transhydrogenase, as well as the IDH2. All these proteins are required for placental steroidogenesis [2,3].

In addition, creatine kinase, hexokinase, VDAC, and HSP60 were identified in the 590-SCS, which have been reported as proteins of contact sites [12]. Other proteins identified in the 590-SCS complex were divided into the following metabolic groups: Krebs cycle, electron transport chain, ATP synthase, and lipid metabolism. Besides, several

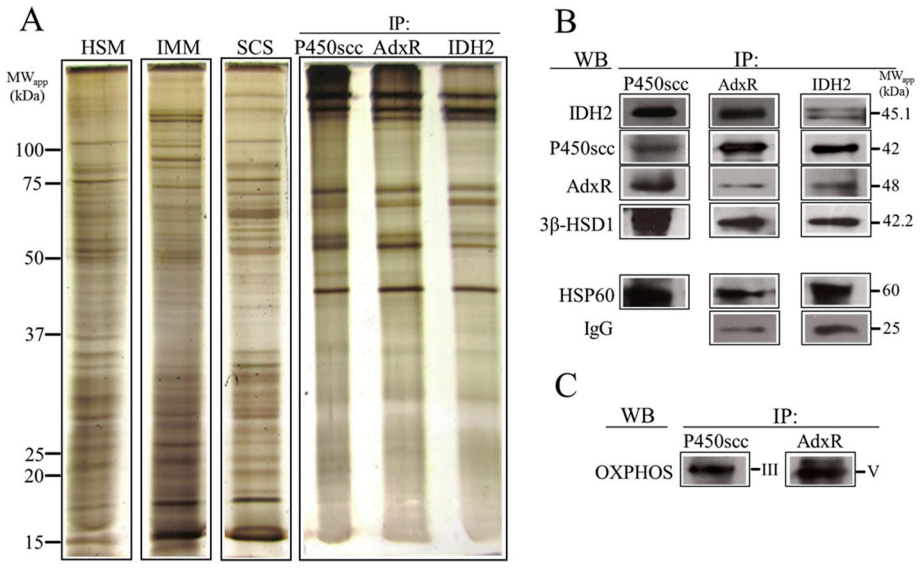


Fig. 3. Electrophoretic pattern of HSM, IMM SCS and IP obtained with different antibodies and identification of proteins by western blot. **A**) Differences among the electrophoretic pattern from HSM, IMM, and SCS, as well as of samples from immunoprecipitation with P450scc, AdxR, and IDH2. **B**) Immunodetection of some proteins associated with P450scc and contact sites and with **C**) oxidative phosphorylation (OXPHOS), where III: UQCR (48 kDa) and V: ATP5A (54 kDa). HSM: Human syncytiotrophoblast mitochondria; IMM: inner mitochondrial membrane; SCS: steroidogenic contact site. IP: immune precipitation. These are representative experiments performed in triplicate from independent samples.

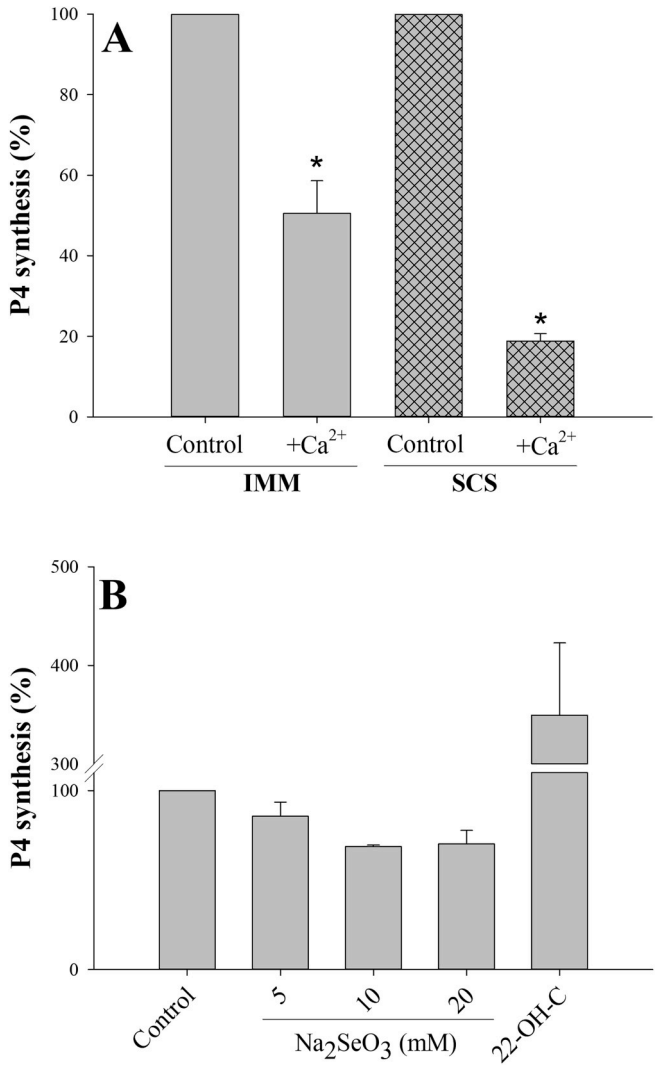


Fig. 4. Progesterone synthesis. **A**) The P4 synthesis was performed in SCS and IMM, incubated in the presence or absence of 8 nM of free Ca^{2+}. **B**) Synthesis of P4 in SCS was determined with increasing concentrations of Na_2SeO_3. 22-hydroxy cholesterol (10 μM) was used as control of the total capacity of steroidogenesis. The data shown represent the mean ± SD of three independent experiments in triplicate from three different samples. For Na_2SeO_3 results are the average of two experiments. (*) represent a statistical difference with control ($p < 0.001$).

proteins were identified as carriers and heat shock proteins (Table 1).

3.3. Western blot of proteins associated with steroidogenesis in SCS and HSM

Since the protein content in the 590-SCS was low, immunodetection of proteins in SCS revealed an increase of the IDH2, P450scc, and HSP60, in comparison to HSM, IMM or OMM (Fig. 2A). AdxR and 3β-HSD1 in SCS were higher as compared to HSM (Fig. 2A). The SCS were also enriched with proteins from oxidative phosphorylation from the electron transport chain associated with the synthesis of ATP (Fig. 2B).

3.4. Association of IDH2 with steroidogenic proteins by immunoprecipitation

In the following experiment, the immunoprecipitation of SCS was performed to identify the possible steroidogenic proteins associated with IDH2. As observed in Fig. 3A, the electrophoretic pattern shows an important decrease of proteins in the immunoprecipitate samples. When some of the proteins related to steroidogenesis were assayed, the results showed that IDH2 was associated to P450scc, AdxR, 3β-HSD1. This result suggests that IDH2 is part of the multiprotein complex.

3.5. Progesterone synthesis

As it has been described, in the HSM, that Ca^{2+} inhibits the IDH2 activity associated with a decrease in P4 synthesis [15], the transformation of cholesterol into P4 was determined in both IMM and SCS. As observed in Fig. 4A, Ca^{2+} inhibited 50% the P4 synthesis in IMM and 80% in SCS. On the other hand, IDH2 is inhibited by Na_2SeO_3. To confirm the role of IDH2 in steroidogenesis, P4 synthesis was determined in SCS in the presence of Na_2SeO_3. The P4 synthesis was decreased as the concentration of Na_2SeO_3 increased in the incubation medium (Fig. 4B). An increase in the P4 synthesis of more than 3-times was observed in the presence of 22-OH-C (Fig. 4B), suggesting that SCS have the machinery to transform cholesterol into P4.

4. Discussion

The reducing equivalent (NADPH) required for placental steroidogenesis is mainly supplied by the mitochondrial IDH2 [15,25,26]. Because of the prominent role of P4 to maintain pregnancy, the production of NADPH becomes relevant because a P4 decrease induces abortion [27].

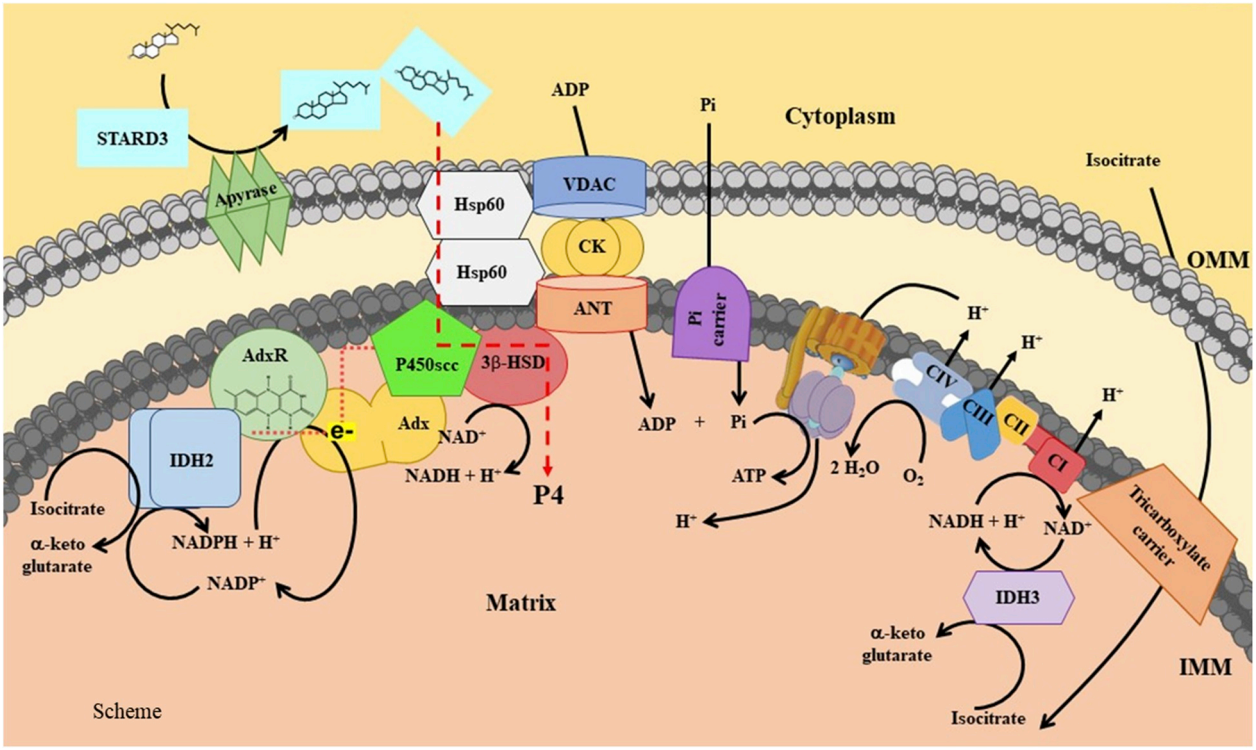
The effectivity of several biological processes requires the association among proteins in suprastructures named metabolomes or transduceosomes [28,29]. The conformation of a multiprotein complex denominated steroidome [30] or steroidosome [31] has been proposed for mitochondrial steroidogenesis.

The electron flux from isocitrate to pregnenolone starts with IDH2 activity, which produces the NADPH to support AdxR activity. The IDH2 is a soluble enzyme of 50 kDa located at the mitochondrial matrix [32]. Surprisingly, IDH2 activity was associated with a ~590 kDa protein complex in the hrCN-PAGE (Fig. 1), probably through hydrophobic or electrostatic interactions, but no the IDH3 (Fig. 1D).

It is worth to point out, that this gel was performed under native conditions, where proteins maintain their associations and structure. Also, it is necessary to comment that the isolation of mitochondria, as well as the method used to obtain OMM, IMM, and SCS were performed under mild treatment, in that way the multiprotein complexes were preserved [12]. This conclusion is supported on data from the MS of the 590-SCS, which reveal several proteins from different metabolic pathways like ATP synthesis, Krebs cycle, or lipid metabolism (Table 1). Proteins from other metabolic processes were also reported by MS in the 590-SCS, such as for carbohydrate metabolism or protein synthesis, which were not analyzed in this study.

It should be taken in consideration, that isolation of the HSM followed several steps of centrifugation and washing the samples to eliminate contamination with other cellular membranes (Supplemental Fig. 1), as previously suggested [33].

The proteins reported by MS in the 590-SCS, as well as the western blot and the immunoprecipitation of SCS, demonstrate that mitochondrial membranes maintained multiprotein complexes conforming contact sites. This information is relevant because it demonstrates that the association among proteins remains even after the physical method of SCS isolation or the hrCN-PAGE treatment, in which IDH2 activity was retained in the band of 590 kDa, where proteins involved in the steroidogenesis such as the P450scc, AdxR and 3β-HSD1 or proteins related to contact sites, like HSP60, creatine kinase, and others were present and are required for placental steroidogenesis [33]. This could be interpreted as the conformation of the steroidosome assuring that cholesterol reaches the P450scc for P4 synthesis at a sufficient production to maintain pregnancy. In addition, these data support that the conformation of multiprotein complexes is vital to maintain efficiently the



Scheme 1. Proposed model of steroidogenesis in the SCS. The model shows the possible localization of proteins implicated in steroidogenesis and ATP synthesis, according to the results presented. The slash line indicates the path of cholesterol for its transformation to P4, whereas the dotted line indicates the electron transport chain coupled to cytochrome P450scc. STARD3: StAR Related Lipid Transfer Domain Containing 3; HSP60: heat shock protein 60; IDH2: isocitrate dehydrogenase type 2; AdxR: adrenodoxin reductase; Adx: adrenodoxin; P450scc: cytochrome P450scc; 3β-HSD: 3β-hydroxysteroid dehydrogenase type 1; VDAC: voltage dependent anion channel; CK: creatine kinase; ANT: adenine nucleotide translocase; CI: electron transport complex I; CII: electron transport complex II; CIII: electron transport complex III. CIV: electron transport complex IV. OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane.

cellular function of the biological processes, in this case, those of the human placenta.

IDH2 is a protein with no transmembrane segments, similar to the Adx; but these two proteins were identified as part of a supramolecular complex with steroidogenic activity. This suggestion is supported on the data observed in P4 synthesis by SCS, since a transformation of cholesterol into P4 was observed. This implicates that IDH2 transformed the added $NADP^+$ into NADPH, which is required to transform cholesterol into pregnenolone, where Adx transfers the electrons between the AdxR and P450scc. Besides, the content of cholesterol was enough in the SCS to support this transformation since no extra cholesterol was added to the incubation medium. However, an increase of more than 3-times of P4 synthesis was observed when 22-OH-C was present in the incubation medium; 22-OH-C is a permeable lipid substrate used frequently to determine the maximal steroidogenic capacity of the electron transport chain coupled to P450scc [34]. These data demonstrate the full capacity of the contact sites to transform cholesterol into P4. The 3β-HSD1 was also present in the SCS, since the transformation of pregnenolone into P4 requires the 3β-HSD1 activity, and an increase in P4 was observed in the presence of 22-OH-C.

On the other hand, both Ca^{2+} and Na_2SeO_3, inhibitors of IDH2 [15,35], decreased the synthesis of P4, supporting the role of IDH2 in placental steroidogenesis associated with SCS. Based on a previous report [15], it could be proposed that Ca^{2+} regulates mitochondrial steroidogenesis in the placenta by modulating the IDH2 activity.

Although proteins reported as members of the SCS [12] where identified through the different methods employed in this work, proteins of other mitochondrial metabolic processes were also identified through MS. In this context, proteins from OMM and IMM found in SCS are consistent with the mechanism probably used by syncytiotrophoblast mitochondria for steroidogenesis, such as proposed in Scheme 1. The two main processes of placental mitochondria, production of P4 and ATP, should be constant and controlled to maintain pregnancy until delivery. It is probable that P4 and ATP synthesis take place at the same time, being oxidizable substrates the mechanism to distribute the production of the reducing equivalent, the NADPH, for steroidogenesis and NADH for ATP synthesis.

Although Scheme 1 shows isocitrate as the main substrate of the reduced equivalent for steroidogenesis, other alternatives should be taken in consideration, since the placental functions are multiple. For example, the human placenta requires high production of ATP to be used in the transport of nutrients from mother to fetus, to release waste from fetus to mother, to synthetize several proteins released from the placenta to mother and fetus to maintain pregnancy, as well as for the ACTH-like hormone or insulin-like proteins [36], among other functions. In this sense, the mitochondrial production of NADH also takes relevance for ATP synthesis, and further research will be necessary to explore this bioenergetic process.

Interestingly, proteins reported to be relevant for steroidogenesis are not present in the SCS or the 590 band, such as the TSPO [37] or MLN64 [14,38], whereas others proposed to form contact sites are described in the MS, such MICOS (Table 1), which could be participating in the mitochondrial cholesterol transport. These data strongly suggest that the protein organization of mitochondria for steroidogenesis is complex and is probably distributed in precise mitochondrial sites with proteins related to specific functions, such as microdomains, which are required, in this case, to maintain pregnancy. In this sense, microdomains in mitochondria have been described as being required to produce cAMP [39]. It will be interesting to determine how these different microdomains interact within the same organelle or with other subcellular domains to maintain the cellular functions as an integrated activity to survive.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.09.008.

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