Optic Atrophy 1-Dependent Mitochondrial Remodeling Controls Steroidogenesis in Trophoblasts

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Summary

During human pregnancy, placental trophoblasts differentiate and syncytiotrophoblasts that sustain progesterone production [1]. This process is accompanied by mitochondrial fragmentation and cristae remodeling [2], two facets of mitochondrial apoptosis, whose molecular mechanisms and functional consequences on steroidogenesis are unclear. Here we show that the mitochondrial shaping protein Optic atrophy 1 (Opa1) controls efficiency of steroidogenesis. During syncytialization of trophoblast BeWo cells, levels of the protein Opa1 and mitochondrial mitofusin (Mfn) 1 increase, and those of Opa1 and mitofusin (Mfn) 2 control mitochondrial fusion and cristae remodeling. Manipulation of the levels of Opa1 reveal an inverse relationship with the efficiency of steroidogenesis in trophoblasts and in mouse embryonic fibroblasts where the mitochondrial stereogenic pathway has been engineered. In an in vitro assay, accumulation of cholesterol is facilitated in the inner membrane of isolated mitochondria lacking Opa1. Thus, Opa1-dependent inner membrane remodeling controls efficiency of steroidogenesis.

Results and Discussion

Steroidogenesis gives rise to multiple lipid hormones, which influence gestation (progesterone), sexual characters (androgens and estrogens), ion homeostasis (mineralocorticoids), and stress response (glucocorticoids) [3]. All stereogenic pathways begin in the inner mitochondrial membrane (IMM) where cytochrome p450sc (Cyp11a1) transforms cholesterol to pregnenolone [4]. The stereogenic acute regulatory protein (Star) transports cholesterol to the IMM, allowing the initiation of steroidogenesis with the remarkable exception of human placenta, where Star is not expressed. In syncytiotrophoblast, the stereogenic cells of human placenta, progesterone synthesis coincides with structural mitochondrial changes that could facilitate cholesterol diffusion to the IMM [2]. We therefore set out to address whether and how the mitochondria-shaping machinery influences steroidogenesis.

Mitochondria Remodel during Syncytialization of Cytotrophoblasts

The human cytotrophoblast BeWo cell line, upon cyclic AMP level increase triggered by the adenylyl cyclase activator forskolin (Frk), can differentiate to syncytiotrophoblast, recapitulating the in vivo differentiation of villous trophoblast [5]. BeWo cells indeed formed multinucleated syncyta when treated with Frk, as revealed by immunostaining for the cell junction marker E-cadherin (Figure 1A). This morphological signature of differentiation was accompanied by a progressive increase in the secretion of the key syncytiotrophoblast pregnancy hormone human chorionic gonadotropin (hCG) [1] (Figure 1B), confirming the suitability of BeWo cells as a model of syncytiotrophoblasts differentiation.

During syncytiotrophoblast formation, mitochondria undergo structural changes that we wished to verify in our cellular model. Confocal imaging of undifferentiated BeWo cells engineered to stably express a yellow fluorescent protein targeted to mitochondria (mtYFP; BeWo-mtYFP) showed elongated mitochondria. Syncytialization and differentiation induced by Frk resulted in mitochondrial fragmentation with the appearance of punctuate, round-shaped organelles (Figures 1C and 1D). Electron microscopy (EM) further elucidated that these morphological changes were accompanied by the appearance of more electron-transparent matrix and by the close apposition of the inner to the outer mitochondrial membrane (OMM) (Figure 1E). A morphometric analysis confirmed that in syncytialized BeWo the number of cristae per mitochondrion was reduced by ~20% (Figure 1F). Z stacks of confocal images of mitochondrial morphology in explants from human second trimester placenta stained with the supravital mitochondrial fluorescent dye nonyl acridine orange indicated a similar elongated to fragmented transition in cyto versus syncytiotrophoblasts in vivo (Figure 1G; overall imaging of villi in 1H).

In order to address the molecular mechanism responsible for the observed fragmentation and reduction in cristae number, we interrogated the levels of the core mitochondria-shaping proteins. In mammals, fission is accomplished by dynamin related protein 1 (Drp1), which oligomerizes on mitochondria and constricts them [6]. Outer membrane (OMM) mitofusin (Mfn) 1 and 2 control mitochondrial fusion [7, 8] together with Optic atrophy 1 (Opa1), an IMM protein that also governs shape of the cristae [9, 10]. In differentiated BeWo cells that express cytochrome Cyp11a1, levels of Opa1 and Mfn2 were greatly decreased, whereas Mfn1 was less reduced and Drp1 was increased ~4-fold. Notably, levels of the respiratory chain component CoxIV and of the OMM protein Fis1 remained stable, indicating a stable mitochondrial mass during differentiation (Figures 1I and 1J). Thus, mitochondria fragment and levels of the mitochondria-shaping proteins change during differentiation from cyto to syncytiotrophoblasts in vitro and in vivo.
Increased Availability of Cholesterol for Mitochondrial Pregnenolone Biosynthesis in Syncytiotrophoblasts

During syncytialization, we also measured an increase in the release of pregnenolone (Figure 2A), which was synthesized by Cyp11a1 as confirmed by the specific inhibitor aminoglutethimide (Figure 2B). Steroidogenesis might be influenced not only by the expression of the key steroidogenetic enzymes but also by the delivery of cholesterol to the IMM where Cyp11a1 is located. To verify this possibility, we compared the basal rate of pregnenolone synthesis with the maximal one, obtained by feeding cells with the membrane-permeable analog of cholesterol and Cyp11a1 substrate 22(R)-hydroxycholesterol (HCH) that freely diffuses to the IMM [11]. The maximal rate depends therefore only on Cyp11a1 levels, whereas the basal one reflects both Cyp11a1 levels and delivery of cholesterol to the IMM. When we fed undifferentiated BeWo cells with HCH, pregnenolone production increased approximately 15-fold, indicating that the ability...
Figure 2. Genetic Analysis of the Role of Mitochondria-Shaping Proteins in Efficiency of Pregnenolone Production during Differentiation of BeWo Cells

(A) Secretion of pregnenolone was measured at the indicated times from cells treated as shown. Where indicated, cells were fed with 22(R)-hydroxycholesterol (HCH). Data represent mean ± SEM of four independent experiments.

(B) Cells were treated with ethanol or Frk for 2 days. Where indicated, 2 hr before the collection of medium for pregnenolone measurements, cells were pretreated with DL-aminogluthethimide (AMG, 1 mM). Data represent mean ± SEM of three independent experiments.

(C) The ratio between basal and maximal pregnenolone synthesis ($R_{\text{preg}}$) determined as in (A) was calculated. Data represent mean ± SEM of four independent experiments.

(D) $R_{\text{preg}}$ was determined in BeWo cells transfected as indicated and treated where indicated with Frk for 48 hr. Data represent mean ± SEM of seven independent experiments. *p < 0.05 in a two-tailed Student’s t test between scramble and single siRNA; #p < 0.05 in a two-tailed Student’s t test between double siRNA against Mfn2 plus the indicated mitochondria-shaping protein and the corresponding single siRNA.

(E) Three-dimensional reconstructions of stacks of confocal images of lipid droplets (green) and mitochondria (red) in BeWo cells transfected with the siRNA against the indicated gene. Insets have been magnified 9×. Where indicated, cells have been treated for 2 days with Frk. Scale bar represents 20 μm.

(F) Quantification of interaction data from (E). Data represent mean ± SEM of four independent experiments.

See also Figures S1–S3.
of cholesterol to reach the IMM (where Cyp11a1 is located) is a rate limiting step in pregnenolone synthesis. Upon Frk-induced differentiation, HCH-stimulated pregnenolone production was doubled (Figure 2A), indicating that the measured induction in Cyp11a1 (Figures 1I and 1J) accounts for a 2-fold increase in the production of pregnenolone. We next moved to the analysis of pregnenolone production by BeWo cells in the absence of exogenous permeable cholesterol. Released pregnenolone levels increased up to 6-fold upon differentiation (Figure 2A). Together with the HCH experiment, these results suggest that differentiation not only induces the expression of the pregnenolone biosynthetic enzymes (Figures 1I and 1J) but also (and perhaps principally) increases IMM cholesterol availability. Accordingly, the ratio between basal and maximal pregnenolone production (Rpreg) tripled during differentiation (Figure 2C). In conclusion, the increased steroiogenic ability of syncytiotrophoblasts is accompanied by an increase in Rpreg that could reflect the increased cholesterol trafficking to the IMM.

Levels of OPA1 Influence Mitochondrial Pregnenolone Biosynthesis
To address whether the mitochondrial shape changes observed during differentiation of BeWo cells were causally linked to pregnenolone synthesis, we efficiently silenced Opa1, Mfn1, Mfn2, Drp1, and the cristae junction regulator mitofilin, and we overexpressed low levels of wild-type murine OPA1 isoform 1 OPA1 (WT), constitutively active disassembly-resistant Q297V [12, 13], and inactive R905stop mutants of OPA1 [9] (Figures S1A–S1C available online). As expected, silencing of profusion proteins caused nonadditive mitochondrial fragmentation at steady state that could not be further aggravated by differentiation, whereas silencing of Drp1 resulted in elongation that was partially retained upon Frk treatment but abolished by the simultaneous silencing of Opa1 (Figures S1D and S1E). Expression of WT and Q297V OPA1 induced the expected mitochondrial elongation, whereas OPA1 R905stop caused slight mitochondrial fragmentation (Figures S1F–S1H). Interestingly, the only two genetic manipulations that affected Rpreg upon BeWo cells differentiation were the ablation of Opa1 (which increased it) and that of Mfn2 (which conversely reduced it) (Figure 2D). The influence of Opa1 on efficiency of pregnenolone biosynthesis was confirmed in the overexpression experiments: WT and constitutively active OPA1 decreased Rpreg, whereas OPA1 R905stop increased it (Figure S2). The stimulation of Rpreg caused by the downregulation of Opa1 could not be counteracted by the simultaneous ablation of Drp1 but was fully inhibited by the cosilencing of Mfn2, which has an additional role in tethering mitochondria to the endoplasmic reticulum (ER) [14] (Figure 2D). The observed changes in Rpreg were not secondary to an overall alteration of the differentiation program, as testified by the lack of effect of the genetic maneuvers listed above on the secretion of hCG (Figure S3).

Ablation of Mfn2 was the only treatment that per se reduced Rpreg, suggesting a key role for this bifunctional mitochondria-shaping protein in the regulation of pregnenolone biosynthesis. Steroidogenesis is mainly fueled by cholesterol imported via low-density lipoproteins (LDL) and stored in lipid droplets, hydrophobic cytoplasmic structures covered with a single leaflet of polar lipids that originate from the ER [15] and that may therefore harbor ER proteins. In addition, ER per se participates in cholesterol trafficking and could therefore provide cholesterol for steroidogenesis. In BeWo cells, the fluorescent NBD-cholesterol analog indeed brightly labeled spots corresponding to lipid droplets, and to a lesser extent the ER (not shown). We therefore addressed whether Mfn2 was involved also in tethering of mitochondria to lipid droplets by analyzing pseudo-colocalization of mitochondria labeled with Mitotracker Red and lipid droplets labeled with BODIPY 493/503. Under basal conditions we observed limited contacts between mitochondria and lipid droplets, which were significantly reduced upon differentiation when levels of Mfn2 decline. Interestingly, ablation of Mfn2 almost completely abolished juxtaposition between mitochondria and lipid droplets to a level that could not be further diminished by Frk (Figures 2E and 2F). Taken together these data suggest a role for Mfn2 in lipid droplet-mitochondria contact, although it is not clear whether this contact could contribute efficiently to steroidogenesis because of its low frequency. Interestingly, defects in ER-mitochondria lipid transports had been indeed suggested in Mfn2−/− MEFs [16] and mice lacking MFN2 display a placental defect [17]. Our results could offer a molecular explanation to this placental deficiency: the decreased tethering between ER or lipid droplets and mitochondria in Mfn2−/− trophoblasts could impair progesterone biosynthesis that is particularly crucial during midgestation in rodents [18].

The combination of the genetic approaches listed here indicates that Opa1 influences pregnenolone biosynthesis, pointing to a role for cristae shape. Indeed, during differentiation of BeWo cells, the IMM remodels ([2] and Figure 1), similar to the loss of cristae observed in syncytiotrophoblasts [2, 19].
the lack of Opa1 to levels comparable to those achieved by the cholesterol shuttle Star in engineered nondifferentiating and nonsteroidogenic cells.

An In Vitro Assay Reveals Increased Cholesterol Loading in Opa1<sup>−/−</sup> Mitoplasts

Several factors other than an increased efficiency of cholesterol shuttling between OMM and IMM could provide the molecular basis for the higher efficiency of pregnenolone biosynthesis upon downregulation or ablation of Opa1. We therefore devised an in vitro assay to directly measure the translocation of fluorescently tagged 22-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-23,24-bisnor-5-cholesten-3β-ol (NBD-cholesterol) to the IMM of isolated mitochondria (Figure 4A). Increasing concentrations of the detergent digitonin solubilized to comparable levels the OMM of Opa1<sup>−/−</sup> and Opa1<sup>−/−</sup>::OPA1 mitochondria loaded with NBD-cholesterol, as revealed by the disappearance of the OMM marker TOM20 and the enrichment in the IMM marker complex III (Figure 4B). In order to verify the influence of OMM stripping on IMM structure, we turned to an assay of cytochrome c release that we successfully employed to determine the amount of cytochrome c escaping from the cristae in the intermembrane space [20]. While stripping of the OMM resulted in complete cytochrome c release from Opa1<sup>−/−</sup> mitochondria, a fraction of it was retained in Opa1<sup>−/−</sup>::OPA1 mitochondria, consistent with the role of OPA1 in cristae retention of cytochrome c [10] and with the stabilization of IMM structure in Opa1<sup>−/−</sup>::OPA1 mitochondria (Figure 4C). Next we compared the amount of NBD-cholesterol as determined by its fluorescence in the lipid extracts from whole mitochondria and mitoplasts. While loading of Opa1<sup>−/−</sup> and Opa1<sup>−/−</sup>::OPA1 mitochondria with NBD-cholesterol was comparable, the concentration of NBD-cholesterol was higher in Opa1<sup>−/−</sup>::OPA1 mitoplasts (Figure 4D), suggesting that in the absence of OPA1 more cholesterol is incorporated in the IMM. This corroborates our hypothesis that OPA1 controls the flux of cholesterol to the IMM, perhaps as a consequence of the structural remodeling of the membrane. It remains unclear whether Opa1 is per se a negative regulator of cholesterol shuttling, or whether these

![Figure 3](current_biology_vol22_no13_1232.png)
changes reflect the structural rearrangements of the IMM controlled by Opa1. Ablation of Opa1 could override the segregation of membrane proteins between the boundary inner membrane and cristae [21–23]. Interestingly, adrenal Cyp11a1 localizes to cristae [22]: it is tempting to speculate that in trophoblasts Cyp11a1 could be exposed to the OMM upon Opa1-controlled cristae remodeling.

Conclusions

Our data extend the involvement of mitochondria-shaping proteins beyond cell death, autophagy, spine plasticity, and cell migration to steroidogenesis. This novel pathway based on the delivery of cholesterol to Star and to its transport to the IMM acts in parallel to Star and can substitute it in a volume of isolation buffer equal to that of the supernatant. Equal volumes of fractions were separated by SDS-PAGE and immunoblotted for cytochrome c.

(D) Mitochondria from MEFs of the indicated genotype were treated as described in (A) and the concentration of NBD-cholesterol was determined in lipid extracts from total mitochondria and from mitoplasts. Data represent mean ± SEM of four independent experiments.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.cub.2012.04.054.

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