Mitochondrial Transcription Factor A (TFAM) is Increased in Rat Embryo During Placentation and Associated with Mitochondrial Differentiation

Maria Pilar Alcolea, Bartomeu Colom, Isabel Lladó, Magdalena Gianotti and Francisco J. García-Palmer

Grup de Metabolisme Energètic i Nutrició. Departament de Biologia Fonamental i Ciències de la Salut. Institut Universitari d'Investigació en Ciències de la Salut (IUNICS). Universitat de les Illes Balears, Palma de Mallorca, Spain

Key Words

Embryo • Mitochondrial subpopulations • Mitochondrial biogenesis • Mitochondrial DNA • Mitochondrial single strand DNA binding protein • Mitochondrial transcription factor A • Cytochrome c oxidase subunit I

Abstract

In the current study, the mitochondrial proliferation-differentiation process was investigated in rat embryo during the placentation process, straight after organogenesis, when there is an important oxidative metabolism activation. For this purpose, on gestational days 11, 12 and 13 we studied the mitochondrial DNA (mtDNA) content and the relative gene expression of proteins involved in mtDNA replication (mitochondrial single strand DNA binding protein (mtSSB)), mtDNA transcription (mitochondrial transcription factor A (TFAM)), as well as in mitochondrial function (cytochrome c oxidase subunit I (COXI)). The results indicated that during placentation important changes in mitochondrial proliferation-differentiation process take place in rat embryo. There is a great decrease in cellular mtDNA content and a rise in the ratio between TFAM and

mtDNA accompanied by an increase in COXI gene expression. Thus, we can conclude that on gestational day 13 mitochondrial differentiation predominates over mitochondrial proliferation in embryo cells. Besides, our work reveals that in a physiological condition such as embryonic development the TFAM levels change in order to regulate the transcriptional activity of mtDNA.

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Introduction

Mitochondria are the main energy production organelles in most eukaryotic cells [1-3], thus alterations in their biogenesis or function are involved in an extensive variety of pathological processes [4]. Mitochondriogenesis is a complex event that includes both mitochondrial proliferation and differentiation [5, 6], and requires the coordinated contribution of both mitochondrial and nuclear genome [7, 8]. The regulation of these two separated genomes is carried out by nuclear transcriptional activators and coactivators that modulate the expression of nuclear genes encoding the necessary proteins for maintenance, replication and expression of mtDNA, as well as, for most

OXPHOS subunits [9, 10]. Among these genes there are those that encode mitochondrial transcription factor A (TFAM) and mitochondrial single strand DNA binding protein (mtSSB). TFAM is one of the regulatory factors necessary for proper mtDNA transcription [11-17]. This protein has a concentration-dependent function, showing higher affinity for the mtDNA light strand promoter than for the heavy strand one [7, 14, 18, 19]. Moreover, TFAM has another reported function, i.e., mtDNA packaging [20-22]. mtSSB is involved in mitochondrial replication by stimulating mtDNA unwinding, stabilizing single strands of mtDNA and enhancing the rate of DNA polymerase activity, among other functions [23-26].

Mitochondrial biogenesis takes place according to specific energy demands in response to several physiological conditions [27-29], including pre- and postnatal developmental stages [5, 30-33]. In this way, the mitochondria of rat embryo undergo considerable morphofunctional changes throughout organogenic period [34-36], coinciding with the switch from glucolytic to oxidative metabolism that takes place on gestational day 12 [35-37], when the nutritional function of visceral yolk sac is transferred to chorioallantoic placenta [38]. Thus, the rat organogenic period is a suitable model to further understand the mitochondrial proliferation-differentiation process.

Previous studies have shown that whole mitochondrial population can be fractionated by differential centrifugation into several subpopulations, which are different in morphological and functional features. A connection between mitochondrial biogenesis and the mitochondrial fractions has been suggested, which would imply that the lightest mitochondria would act as transitional forms of the heaviest, i.e., more differentiated ones [39-44]. This fact renders the mitochondrial subpopulations a suitable tool for the study of mitochondrial biogenesis.

In order to go further into the knowledge of mitochondrial biogenesis in a physiological condition such as development, in the current work we measured the mtDNA content and the relative gene expression of key proteins involved in mitochondrial proliferation (mtSSB) and differentiation (TFAM), in rat embryo mitochondria during the placentation period. Here we reached the conclusion that during the period studied embryo mitochondria reduce their proliferation and enter a differentiation stage by increasing their transcriptional activity probably through the physiological TFAM function as a mitochondrial transcription factor.

Materials and Methods

Materials

Oligonucleotide primer sequences were from Roche Diagnostics (Basel, Switzerland). Antibodies for COXI were obtained from Molecular Probes (A-6403; Oregon, US) and rabbit antisera against TFAM was kindly provided by Dr. H. Inagaki [45].

Chemicals for mRNA reverse transcription were obtained from Applied Biosystems (California, USA). Lightcycler-FastStart DNA Master SYBR Green I for real-time PCR was supplied by Roche Diagnostics (Basel, Switzerland). Chemicals for immunoblot development were provided by Amersham (Little Chalfont, UK).

Routine chemicals used were supplied by Sigma-Aldrich (St. Louise, USA), Roche Diagnostics (Basel, Switzerland), Bio-Rad (California, US), Panreac (Barcelona, Spain) and Pronadisa (Madrid, Spain).

Animals

Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and the EU (86./609/EEC) regulations. Three-month old virgin female Wistar rats, purchased from CRIFFA (Barcelona, Spain), were housed in individual cages at 22 °C with a 12h light/dark cycle (lights on at 08:00 a.m). The rats had free access to tap water and pelleted standard diet (Panlab, Barcelona). Vaginal smears were examined for sperm the morning after overnight mating. When intravaginal sperm was found, day 0 of pregnancy was defined as the preceding midnight.

Sacrifice and isolation of samples

Pregnant rats were sacrificed by decapitation on gestational days 11, 12 or 13. Uteri were removed and half of conceptuses (embryos with intact amniotic and yolk sacs) were dissected into a sterile culture dish with physiological saline solution which contained 0.1% v/v diethyl pyrocarbonate (DEPC) to inactivate RNase activity. Embryos were obtained and were rapidly pooled, weighed, and frozen in nitrogen liquid and stored at -70 °C for their subsequent RNA extraction.

The remaining conceptuses were dissected without DEPC added to saline solution. The embryos were homogenized in isolation buffer (10mM Tris-HCl, 250mM sucrose, 1mM EDTA, pH 7.5) and used for mitochondria isolation. Four to five rats on day 11, three to four on day 12 and two to three on day 13 were used to pool the embryos for sufficiently large samples in each of the six to nine experiments developed.

Isolation of mitochondrial subpopulations

Mitochondrial fractions were obtained according to Justo et al. [44]. In brief, nuclei and cell debris were first removed using a refrigerated centrifuge (Sigma-3K30; St. Louise, USA) at 700xg for 10 minutes at 4°C. The resulting supernatant was subjected to three 10 minute sequential centrifugation steps, at 1000xg, 3000xg, and 8000xg respectively, rendering the heavy (M1), medium (M3) and light (M8) mitochondrial subpopulations. The pellets were resuspended in the homogenizing buffer and frozen at -70 °C for subsequent

Table 1. Oligonucleotide primer sequences used in real-time PCR amplification. mtSSB, mitochondrial single strand DNA binding protein; TFAM, mitochondrial transcription factor A; COXI, cytochrome c oxidase subunit I; 18S, ribosomal RNA 18S.

Gene	GenBank number	Forward Primer (5'-3') Reverse Primer (5'-3')	Product length	T _m
mtSSB	M94557	GCG TCA GGA AAA GCC TAA AG TCG CCA CAT CTC ATT TGT TG	243 bp	84.3
TFAM	AJ 312746	GCT AAA CAC CCA GAT GCA AAA CGA GGT CTT TTT GGT TTT CC	269 bp	84.1
COXI	X14848	AGC AGG AAT AGT AGG GAC AGC TGA GAG AAG TAG TAG GAC GGC	520 bp	84.3
18S	X01117	GAG GTG AAA TTC TTG GAC CGG CGA ACC TCC GAC TTT CGT TCT	93bp	83.0

analysis. Total protein content was measured in both homogenate and mitochondrial fractions [46]. Total DNA content was tested in homogenate samples [47].

mt-DNA extraction and semi-quantification

mtDNA extraction and semi-quantification was carried out in both homogenate and isolated mitochondrial subpopulations as previously described [48]. The extracted mtDNA was quantified by real-time PCR using SYBR Green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-TACACGATGAGGCAACCAA-3' and 5'-GGTAGGGGTGTGTTGTGAG-3'.

Real-time RT-PCR analysis

Total RNA was isolated from frozen embryo samples using TriPure® isolation reagent and quantified using a spectrophotometer set at 260 nm.

One µg of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 µl volume of retrotranscription reaction mixture containing 10 mM tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X®-100, 2.5 mM MgCl₂, 2.5µM random hexamers, 10 U RNase inhibitor and 500µM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (-70 °C) until the PCR reactions were carried out.

Real-time PCR was done for three target genes: mitochondrial single strand DNA binding protein (mtSSB), mitochondrial transcription factor A (TFAM) and cytochrome c oxidase subunit I (COXI); and one housekeeping gene: 18S ribosomal RNA (18S). All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0. Finally, a basic local alignment search tool (NCBI Blast) revealed that the primer sequence homology was obtained only for the target genes.

Real-time PCR was performed using SYBR Green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Each reaction contained 1 µl Lightcycler-FastStart DNA Master SYBR Green I (containing FastStart Taq DNA polymerase, dNTP mix, reaction buffer,

MgCl₂ and SYBR Green I dye), 0.5 µM of the sense and antisense specific primers (Table 1), 2mM MgCl₂ (for all, except for COXI which was 3mM MgCl₂) and 3 µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (10 min 95 °C), followed by 40 cycles consisting of a denaturation step (10 s. 95 °C), an annealing step (10 s. 58 °C for mtSSB, 60 °C for TFAM and 62 °C for 18S; 8 s. 55 °C for COXI) and an extension step (12 s. 72 °C for all, except for COXI which was 26 s. 72 °C). After each cycle, fluorescence was measured at 72 °C. A negative control without cDNA template was run in each assay.

The real-time PCR efficiencies (e) were estimated on average of all sample efficiencies, which were calculated by the following formula: $e = (F/F_0)^{1/n-n_0}$, where n and n₀ were the crossing point values of F and F₀ respectively, fluorescences that belong to the linear section of the logarithmic quantification curve.

Western-blot analysis for mitochondrial transcription factor A (TFAM) and cytochrome c oxidase subunit I (COXI)

Equal amounts of mitochondrial protein (35 µg) were electrophoresed on SDS-PAGE gels containing 15% v/v polyacrilamide. Proteins were electrotransferred onto a nitrocellulose filter and Ponceau S staining was performed systematically to check the correct loading and electrophoretic transfer. Blots with mitochondrial proteins were incubated with mouse monoclonal antibody for COXI and rabbit antisera against TFAM. Immunoblot development was performed using an enhanced chemiluminescence western blotting analysis system. Film bands were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). The apparent molecular weights of the proteins detected were 25 kDa and 39 kDa, for TFAM and COX I, respectively [49, 50].

Statistics

All data are expressed as mean values ± SEM. Differences between gestational days in embryo weight and biochemical parameters were assessed by one-way analysis of variance

Table 2. Embryo weight and composition on gestational days 11, 12 and 13. Total protein and DNA content were measured in homogenate samples. Data represents the mean $\pm$ SEM of six to nine independent experiments. One-way ANOVA ($p < 0.05$): D, gestational day effect. Post hoc analysis (LSD test; $p < 0.05$): a = 11 vs 12, b = 11 vs 13, c = 12 vs 13.

Gestation Day		11	12	13	Anova
Embryo weight	(mg)	3.49 $\pm$ 0.29	17.1 $\pm$ 1.4	62.3 $\pm$ 2.0	D _{a, b, c}
Total Protein Content	(mg/ g of embryo)	92.7 $\pm$ 27.7	87.5 $\pm$ 10.7	72.2 $\pm$ 5.2	NS
	(mg/ embryo)	0.331 $\pm$ 0.097	1.45 $\pm$ 0.12	4.46 $\pm$ 0.30	D _{a, b, c}
Total DNA Content	(mg/ g of embryo)	9.20 $\pm$ 1.08	7.42 $\pm$ 0.28	6.15 $\pm$ 0.19	D _{a, b}
	(μ g/ embryo)	31.9 $\pm$ 4.0	117 $\pm$ 6	377 $\pm$ 13	D _{a, b, c}

Table 3. Mitochondrial subpopulation protein content of embryo on gestational days 11, 12 and 13. Protein content was measured in mitochondrial subpopulations M1, M3 and M8 (heavy, medium and light) of embryo. Data represents the mean $\pm$ SEM of six to nine independent experiments. Two-way ANOVA ($p < 0.05$): F, fraction effect; D, gestational day effect. Post hoc analysis (LSD test; $p < 0.05$): a = 11 vs 12, b = 11 vs 13, c = 12 vs 13.

Gestation Day		11	12	13	Anova
mg/ g of embryo	M1	1.93 $\pm$ 0.34	1.44 $\pm$ 0.25	0.89 $\pm$ 0.15	D _{b, c}
	M3	1.59 $\pm$ 0.23	1.72 $\pm$ 0.26	1.06 $\pm$ 0.13	
	M8	1.35 $\pm$ 0.16	1.16 $\pm$ 0.16	0.90 $\pm$ 0.11	
μ g/ embryo	M1	6.73 $\pm$ 1.22	23.3 $\pm$ 3.2	55.8 $\pm$ 10.3	D _{a, b, c}
	M3	5.87 $\pm$ 1.34	28.5 $\pm$ 3.3	66.2 $\pm$ 8.2	
	M8	4.83 $\pm$ 0.87	19.3 $\pm$ 2.2	56.8 $\pm$ 7.8	

(ANOVA) followed by LSD post hoc test. Differences between mitochondrial subpopulations and gestational days were assessed by two-way ANOVA using the statistical software package SPSS 11.0 for WINDOWS (Chicago, IL).

The statistical PCR data analysis was performed using the Relative Expression Software Tool (REST). The statistical model used was a Pair Wise Fixed Reallocation Randomisation Test[®] [51, 52]. Differences in expression between groups were assessed using the means for statistical significance by randomisation tests, a proper model to avoid the normal distribution of the data.

Results were considered statistically significant at the $p < 0.05$ level.

Results

Embryo weight and composition

Embryo weight as well as total protein and DNA content in embryo samples (Table 2) rose exponentially throughout gestational days as expected, due to the considerable growth of the embryo during the period studied. Thus, both cellular hypertrophy and hyperplasia take place during the placentation period.

Total DNA and protein expressed per gram of embryo showed a downward trend throughout placentation, but was only statistically significant in DNA. The scatter of the individual values was rather large,

probably due to the high rate of embryo growth at this particular stage of development, such that small differences in the developmental age of the embryo would result in a high variability of the data.

Mitochondrial subpopulation protein content

In order to study mitochondrial biogenesis, mitochondrial subpopulations were used as a tool. Throughout the gestational days studied, all fractions (M1, M3, M8) showed a decreasing profile when the protein content was expressed per gram of embryo and an important rise when it was expressed per embryo (Table 3), in accordance with previous reports [44]. This profile was similar to that observed above for total DNA in homogenate samples. In fact, there were no significant differences in the ratio between embryo mitochondrial protein content and total DNA (data not shown).

Mitochondrial DNA content

Quantification of mtDNA content was performed in both homogenate and mitochondrial subpopulations of rat embryo (Figure 1 and 2), as an indicator of mitochondrial number and therefore, of mitochondrial proliferation [43, 53].

The ratio between mitochondrial subpopulation mtDNA and total DNA (Figure 2A) showed a fraction

Fig. 1. Rat embryo mitochondrial DNA (mtDNA) content during placentation period. mtDNA content was analyzed in homogenate samples and values on day 11 were set as 1. Data represents the mean $\pm$ SEM of six to nine independent experiments. One-way ANOVA ($p < 0.05$): D, day effect. Post hoc analysis (LSD test; $p < 0.05$), Post hoc day: a = 11 vs 12, b = 11 vs 13, c = 12 vs 13.

decreasing profile, which was significantly lower in the M8 fraction than in the other two, indicating a greater number of heavy mitochondria in embryo cells. However, it has to be taken into account that the mitochondria of the heavy subpopulations have i) a higher protein content [44, 48] and ii) a higher content of mtDNA per equal amounts of mitochondrial protein (Figure 2B). Hence, the most likely hypothesis is that the mitochondria of the heavy fractions have a greater copy number of mtDNA than the light ones, a suggestion that would be in agreement with previous studies performed in brown adipose tissue [48].

Throughout the gestational days studied, the ratio between embryo mtDNA and total DNA in mitochondrial subpopulations (Figure 2A) showed a striking drop, which would indicate a decrease in the number of mitochondria in embryo cells. Such a downward pattern could be due to the great cellular proliferation that takes place during

Fig. 2. Rat embryo mitochondrial DNA (mtDNA) content of M1, M3 and M8 subpopulations during placentation period. mtDNA content of M1 on day 11 was set as 1. Data represents the mean $\pm$ SEM of six to nine independent experiments. tDNA, total DNA; mtP, mitochondrial protein. Two-way ANOVA ($p < 0.05$): F, fraction effect; D, day effect. Post hoc analysis (LSD test; $p < 0.05$), Post hoc day: a = 11 vs 12, b = 11 vs 13, c = 12 vs 13; Post hoc fraction: d = M1 vs M3, e = M1 vs M8, f = M3 vs M8.

placentation, which would exceed the mitochondrial proliferation capacity. Nevertheless, the significant striking drop shown by the ratio between mtDNA and total DNA in homogenate samples on day 13 (Figure 1A), together with the no significant upward trend observed in mtDNA per embryo of both homogenate and mitochondria (Figure 1B and 2C) could indicate a possible reduction in mtDNA replication. Besides, there was a significant gestational day decrease in the ratio between mtDNA and mitochondrial protein (Figure 2B). Thus, all these data

indicate that throughout the period studied the embryo cells reduced their number of mitochondria, but these mitochondria enriched their protein content indicating an activation of mitochondrial differentiation.

Relative mRNA levels of mtSSB, TFAM and COXI

With the purpose of finding out whether the gestation decreasing pattern of the ratio between embryo mtDNA and total DNA is also related to a reduction in mtDNA replication, the mtSSB mRNA relative expression was analysed (Figure 3A), as a protein strongly involved in mtDNA replication [23-26]. Embryo mtSSB mRNA levels showed a significant decrease throughout the period studied (1.7 fold on day 12 and 2.3 fold on day 13, both compared with day 11). These results are consistent with the drop in the number of mitochondria in embryo cells through a decrease in mitochondrial proliferation because of a likely reduction in mtDNA replication.

The mRNA levels of TFAM and COXI were analyzed as indicator of mitochondrial differentiation, since TFAM is a mitochondrial transcriptional factor [8, 11-17] and COXI is a protein encoded by mtDNA heavy strand, which encodes most of the OXPHOS mitochondrial subunits [7].

Throughout the gestational period studied, TFAM mRNA levels (Figure 3B) showed a significant down-regulation (about 1.6 fold decrease for both day 12 and 13 comparing with day 11), a similar profile to those described above for mtSSB. These results are in accordance with previous studies focused on TFAM function [54, 55], in which a similar way of regulation has been proposed for both TFAM and mtSSB genes.

The mRNA expression of COXI (Figure 3C) showed an important up-regulation on day 13 (a rise of 2.1 fold vs day 11 and of 2.4 fold vs day 12). This fact is also in agreement with the idea that during placentation the mitochondria would enrich their protein content, indicating a higher degree of mitochondrial differentiation.

TFAM and COXI Protein Content

Once COXI mRNA levels were found to follow a different profile than its transcriptional factor (TFAM), we decided to study their protein content.

In each embryo mitochondrial subpopulation, TFAM protein levels (Figure 4A) showed a significant increase on gestational day 13 when they were expressed per mitochondrial protein. This rise was more evident when it was expressed per mtDNA (Figure 4B) likely due to the important decrease of mtDNA observed throughout the period studied. The latter could reflect an up-regulation

Fig. 3. Rat embryo mRNA levels of mtSSB (mitochondrial single strand DNA binding protein); TFAM (mitochondrial transcription factor A) and COXI (cytochrome c oxidase subunit I) during placental period. Values on day 11 were set as 100%. Data represents the mean expression of six to nine independent experiments. The statistical model used was a Pair Wise Fixed Reallocation Randomisation Test © ($p < 0.05$): a = 11 vs 12, b = 11 vs 13, c = 12 vs 13.

of mtDNA expression, since TFAM transcriptional function positively depends on its mtDNA ratio up to a molar ratio of about 100 and then decrease [11, 14, 18]. Taking into account that the transcription of all the mRNAs encoded by the heavy strand originates a polycistronic molecule [7], the current TFAM data together with the important COXI mRNA up-regulation seen on day 13, would indicate a likely transcriptional activation of the mtDNA heavy strand by TFAM on this day. In this sense,

Fig. 4. Rat embryo TFAM (mitochondrial transcription factor A) and COXI (cytochrome c oxidase subunit I) protein content of M1, M3 and M8 subpopulations during placentation period. M1 values on day 11 were set as 1. Data represents the mean $\pm$ SEM of six to nine independent experiments. Two-way ANOVA ($p < 0.05$): F, fraction effect; D, day effect. Post hoc analysis (LSD test; $p < 0.05$), Post hoc day: a = 11 vs 12, b = 11 vs 13, c = 12 vs 13; Post hoc fraction: d = M1 vs M3, e = M1 vs M8, f = M3 vs M8.

although we cannot discard that such mRNA up-regulation was due to changes in its stability, it is poor likely since the increase in mitochondrial mRNA stability has been mainly reported as response to compensate the inhibition or decrease of mitochondrial transcription [12, 56, 57]. Thus, on day 13 embryo mitochondrial subpopulations would be in a more differentiated stage compared with the previous days. The decrease in TFAM mRNA levels seen above, together with the increase in its mitochondrial protein content throughout placentation point towards the existence of regulatory mechanisms of gene expression likely to be acting at post-translational level, as has been previously reported [58].

The ratio between mitochondrial TFAM and mitochondrial protein (Figure 4A) showed a fraction decreasing profile, which was significantly higher in heavy subpopulation than the M8 one. Although this fact could indicate a higher differentiation status in the heavy fractions than in the lightest one, it could not be confirmed since this difference disappeared when TFAM was expressed per mtDNA.

In order to find out whether the up-regulation of COXI mRNA levels on gestational day 13 was translated into a rise in COXI protein content and so into an increase in the OXPHOS subunits synthesis, its protein content was assayed in each mitochondrial subpopulation (Figure 4C).

Throughout the gestational days studied, the COXI ratio per both mitochondrial protein and mtDNA (Figure 4C and 4D) showed a similar profile to the one observed for TFAM, i.e., a significant increase on day 13. In fact, there were no significant differences in the ratio between COXI and TFAM throughout the gestational days studied (Figure 4E) but a downward trend, which could be indicative of a backwardness expression of COXI compared to TFAM. These results, together with the important COXI mRNA increase on day 13, would support the idea of a likely activation of the mtDNA heavy strand promoter by mitochondrial transcription factor A,

stimulating the expression of mRNA encoding for mitochondrial proteins, such as COXI, which would lead mitochondria to a more differentiated stage.

Discussion

Rat placentation is a period of great interest in the study of mitochondrial biogenesis because of the important activation of the oxidative metabolism. Our results point out that the changes in TFAM/mtDNA ratio in a physiological condition, such as development, could induce the mtDNA expression and mitochondrial differentiation.

We demonstrate here that during the placentation period, to be precise on day 13, there is a significant decrease in mtDNA content per cell, suggesting, according to Koekemoer [43], a decrease in the number of mitochondria of each subpopulation per embryo cell. This, together with the significant decrease observed in embryo mtSSB mRNA levels, indicate that a reduction of mtDNA replication would take place during placentation, accompanied by important cellular proliferation that would exceed the proliferative capacity of the mitochondria. These results are consistent with those previously described for the developmental period from oocyte to blastocyst, in which the high number of mitochondria formed during oogenesis [30] are distributed among the embryo cells until the blastocyst stage, when mitochondrial replication is resumed [31, 32]. Thus, it seems that during development there would be important growth points that are preceded by a massive amplification of mitochondrial genomes, which will be subsequently distributed between the proliferating cells.

On the other hand, during placentation, embryo mitochondria showed variations in TFAM protein content, which were greater when TFAM was represented per mtDNA due to the important decrease in the latter throughout the period studied. As mtDNA heavy strand encodes most of the mitochondrial protein information [7], the presence of a TFAM/mtDNA ratio high enough to stimulate the high strand promoter transcription could be considered indicative of mitochondrial differentiation [14, 18], provided that the mRNAs are translated into proteins. This is what probably happened in embryo mitochondria on gestational day 13, that is, an increase in mitochondrial TFAM content per mtDNA, which as a mitochondrial transcription factor could be the main cause of the significant rise seen in COXI mRNA gene expression and subsequently in COXI protein content. Thus, embryo mitochondria seem to be in a more

differentiated stage on gestational day 13 compared with the previous days studied.

The present results would indicate the beginning of a mitochondrial differentiation process, at least at a molecular level, in which these organelles would reduce their replication and incorporate more proteins. In fact, this differentiation process is corroborated by the more lamellated appearance and the increase in COX specific activity seen in rat embryo mitochondria during the placentation period [36, 44]. Moreover, if we take into account the increase in protein content observed in embryo mitochondria during placentation we can conclude that these mitochondria are actually enhancing their functionality. The aforementioned is in agreement with the fact that during this developmental stage an important activation of oxidative metabolism is taking place, which will contribute to supply the necessary energy for rat embryo development [35, 36].

Several studies have reported the function of TFAM as a mitochondrial regulatory factor in "*in vitro*" conditions and in some "*in vivo*" treatments with mitochondriogenesis-inducing molecules, such as thyroid hormones [11-17]. To our knowledge, in this study the TFAM regulatory function is first observed in a physiological situation, like embryonic development. Thus, the current TFAM data suggest that the levels of this transcriptional factor and its mtDNA ratio could be modulated in certain physiological situations and that modulation could affect mitochondrial genome expression and, as a result, would affect mitochondrial function.

On the other hand, the present results are in accordance with the mitochondrial subpopulation profile previously observed in undeveloped tissues, in which the lack of important differences between the embryo mitochondria fractions were described [44], unlike well-developed tissues such as brown adipose tissue [48]. Nevertheless, the current results of mtDNA give further support to the idea that mtDNA copy number is greater in the heavy fractions than in the light ones [48], suggesting an active mtDNA replication throughout the mitochondrial differentiation process, which reflects the connection between mitochondrial subpopulations and the mitochondrial proliferation-differentiation process in cells [39-43].

In conclusion, on gestational day 13, rat embryo cells decrease their number of mitochondria most likely due to a reduction of mtDNA replication and also to the great hyperplasia taking place in embryo. Besides, these mitochondria enter a differentiation stage increasing their protein content probably through the physiological TFAM

function as a mitochondrial transcription factor. To sum up, the present study supports the fact that development is a physiological condition where mitochondrial biogenesis is well illustrated. Thus, the current model could be of great interest for further understanding many unknown aspects of mitochondriogenesis, which should help understand the pathophysiology of mitochondrial diseases.

Abbreviations

OXPHOS, oxidative phosphorylation system; mtDNA, mitochondrial DNA; TFAM, mitochondrial transcription factor A; mtSSB, mitochondrial single strand DNA binding protein; COXI, cytochrome c oxidase subunit I; 18S, 18S ribosomal RNA.

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