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The study of mitochondrial biogenesis during fetal development

PhD Thesis

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Praha, 2010

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Identifikační záznam:
PEJZNOCHOVÁ Martina. *Studium mitochondriální biogeneze v průběhu fetálního vývoje. [The study of mitochondrial biogenesis during fetal development].* Praha, 2010. 76 stran, 4 přílohy. Disertační práce. Univerzita Karlova v Praze, 1. lékařská fakulta, Klinika dětského a dorostového lékařství. Prof. MUDr. Jiří Zeman, DrSc.

Abstrakt:

Postnatální adaptace novorozence na extrauterinní prostředí je kromě jiného závislá i na efektivním průběhu mitochondriální biogeneze v období fetálního vývoje. Proto studium mitochondriální biogeneze na molekulárně genetické a biochemické úrovni může přispět k zlepšení péče o předčasně narozené děti, zvláště o kriticky nemocné novorozence.

Práce vznikla v Laboratoři pro studium mitochondriálních poruch Kliniky dětského a dorostového lékařství 1.LF UK v Praze a zabývá se molekulárně genetickými analýzami zaměřenými na charakterizaci exprese genů podílejících se na mitochondriální biogenezi včetně genů pro systém oxidativní fosforylace (OXPHOS) a na charakterizaci změn množství mitochondriální DNA v průběhu fetálního vývoje člověka.

Získané výsledky umožňují:

- Efektivní analýzu expresní hladiny mRNA metodou real-time PCR ve fetálních tkáních
- Kvantifikaci množství mtDNA v různých fetálních tkáních
- Pochopit a vysvětlit tkáňové specifické rozdíly v expresi OXPHOS genů a genů podílejících se na transkripci mtDNA a regulaci jejího množství v průběhu druhého trimestru gestace

Klíčová slova: mitochondrie, mtDNA, biogeneze, fetální vývoj

Abstract:

Postnatal adaptation of neonate to extrauterine life is among others dependent on effective mitochondrial biogenesis during fetal development. Therefore the study of mitochondrial biogenesis on molecular and biochemical level may improve the care of very premature neonates, especially critically ill premature neonates.

This thesis has been worked out in The laboratory for study of mitochondrial disorders (Department of Pediatrics, 1st faculty of Medicine, Charles University in Prague). The thesis is based on molecular genetic analyses, which are focus on characterization of expression of gene involved in mitochondrial biogenesis including gene of oxidative phosphorylation system (OXPHOS) and on changes in mtDNA content during human fetal development.

The results than enable :

- Efective analysis of the mRNA expression level by quantitative real-time PCR method in fetal tissues.
- Analysis of the changes in the mtDNA content in different fetal tissues
- To understand and to explain the tissue-specific differences in expression of the OXPHOS genes and of the genes involved in mtDNA transcription and in regulation of mtDNA content during second trimester of gestation.

Key words: mitochondria, mtDNA, biogenesis, fetal development

ACKNOLEDGEMENTS

I would like to thank to my supervisor Prof. MUDr. Jiří Zeman, DrSc. for the opportunity to participate in the research of fetal OXPHOS maturation and for his intellectual support.

Special thanks belong to RNDr. Hana Hansíková and Ing. Markéta Tesařová for their valuable comments, help and support.

This thesis would not have been possible without friendly atmosphere in our lab. Therefore I thank to all my colleagues from Laboratory for Study of Mitochondrial Disorders.

Last but not least I am grateful for patience and gracious support of my family and of my dear partner.

The PhD thesis was elaborated during Postgraduate Studies in Biomedicine at the 1st Faculty of Medicine of Charles University and Academy of Sciences of the Czech Republic in years 2004-2010 and was supported by GAUK 257557/557/07, GAUK 16/04, Institutional Research Project Center of Applied Genomics 1M6837805002, IGA MZ NR/9410-3, GACR 303/03/H065, GACR305/08/H037, MSM0021620806 and by Institutional Research Project Center of AppliedGenomics 1M0520.

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1 INTRODUCTION

1.1 Mitochondria

Mitochondria are best known for their role as cell “powerhouses” because they generate ATP from metabolic fuels through oxidative phosphorylation system (OXPHOS) (Gibson, 2005; Papa, 1996). Moreover mitochondria are involved in the metabolisms of amino acids, nucleotides and lipids, in the urea cycle, biosynthesis of heme and even in maturation of cellular Fe-S proteins. Further they have important role in ion homeostasis, cell proliferation, motility and apoptosis (Cannino, et al., 2007; Craig, et al., 1999; Lemasters, et al., 2009; Lill, et al., 1999). Therefore these semiautonomous organelles are essential for practically all eukaryotic cells. Different tissues contain mitochondria that differ substantially in number, structure and function, reflecting their variable energetic demand (Garesse and Vallejo, 2001; Heerdt and Augenlicht, 1990).

According to the endosymbiotic theory, mitochondria are the descendents of bacteria. The theory postulates that a primitive cell (archaeon) engulfed some aerobic bacteria (α-proteobacterium) and rather than digest them, a symbiotic relationship was established (Gray, 1999; Poole and Penny, 2007). Major part of mitochondrial genes were transferred to nucleus, therefore mammalian cells had to acquire the capability to take control of regulatory cross-talk between two genomes.

As refer Nisoli et al. (2004), it was Karl Benda (1857-1933) who firstly coined the name mitochondrion, derived from the Greek “mitos”, a thread, and “chondros”, a grain, which describes the appearance of mitochondrion during spermatogenesis. However, Richard Altmann was the first one to recognize the existence of these organelles but called them “bioblasts”. Mitochondria have a complex internal structure and consisted of at least six compartments: outer membrane, inner boundary membrane, intermembrane space, cristal membranes, intracristal space, and matrix (Logan, 2006) (figure 1). On the other hand, their external shape is variable and they are frequently found as extended reticular networks (Soubannier and McBride, 2009). There are continual cycles of mitochondrial fusion and fission, opposing processes that are essential for maintenance of architecture of these organelles (Liesa, et al., 2009). In particular, mitochondrial fusion enables intermitochondrial cooperation by allowing exchange of both membrane and matrix components. Therefore the fusion process may help to restore local depletions and

maintain mitochondrial function. This suggests that a mitochondrial fusion process has an integral role in the biogenesis of functional mitochondria (Nisoli et al., 2004).

The outer membrane encloses the entire organelle and has a protein-to-phospholipid ratio similar to the eukaryotic plasma membrane (about 1:1 by weight). It contains numerous integral proteins called *porins*, which contain a relatively large internal channel (about 2-3 nm) that is permeable to all molecules

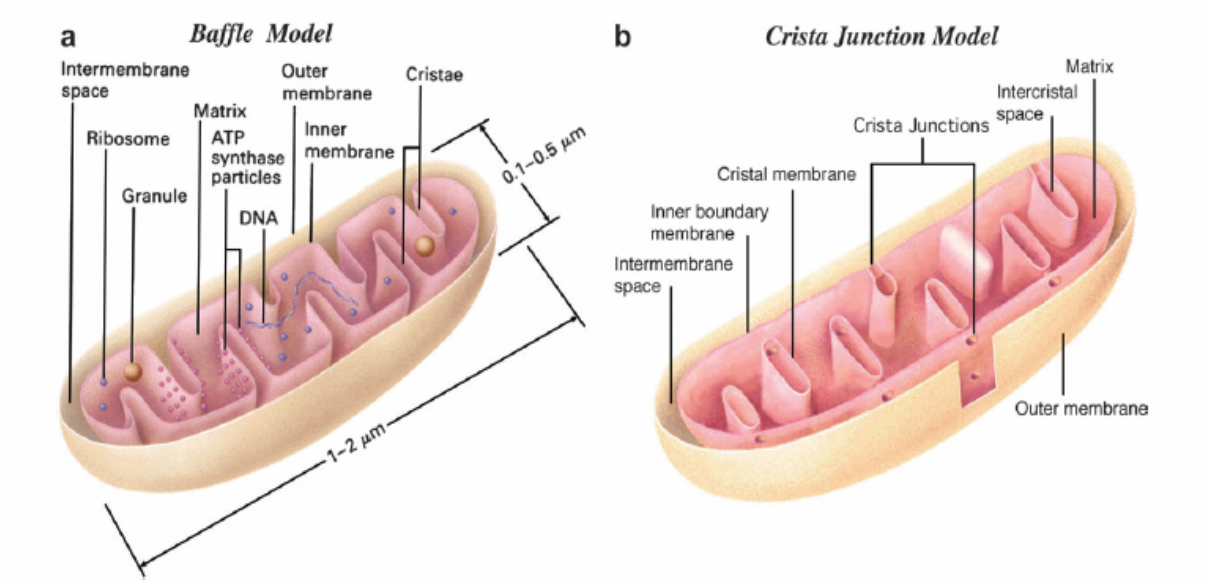

Figure 1. Models of mitochondrial membrane structures. (Logan, 2006)
(a) Baffle model is the best known representation of mitochondrial structure. This model has been prominent until recently. (b) Crista junction model substitutes the baffle model for all mitochondria examined to date from higher animals. Instead of the large openings connecting the intercristal space to the intermembrane space present in the baffle model, narrow tubular openings (crista junctions) connect these spaces in this model.

up to molecular weight of 5000 daltons. Larger molecules can only pass the outer membrane by active transport through mitochondrial membrane transport proteins (permeability transition pore, calcium uniporter, sodium/calcium exchanger) (Crompton, 1999; Rizzuto, et al., 2009). The outer membrane also contains enzymes involved in such diverse activities as the transport of long chain fatty acids (carnitine palmitoyltransferase I) or oxidation of adrenaline (monoamine oxidase) (Stryer, 1988).

The inner membrane is loosely permeable only to oxygen, water and carbon dioxide. Its structure is highly complex, including systems used to transport reducing agents, which are named mitochondrial shuttles (glycerol-phosphate

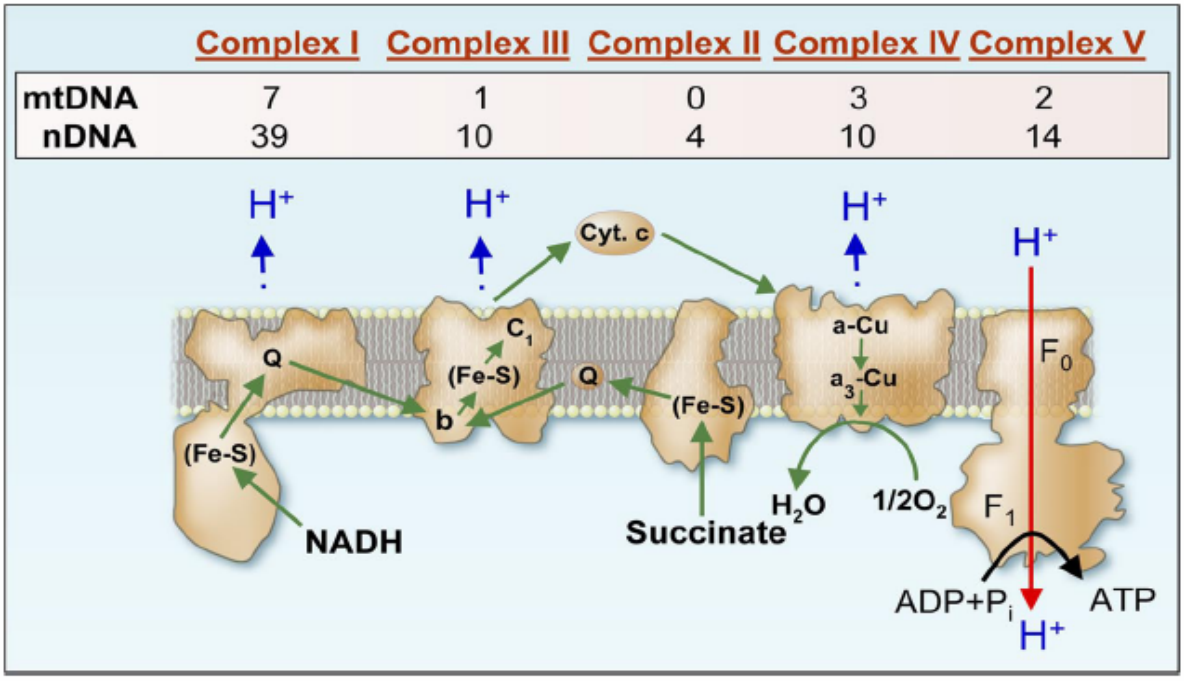

Figure 2. Summary of protein subunits of the OXPHOS complexes encoded by nuclear and mitochondrial genes (Scarpulla, 2008b)
Green arrows show the pathway of electrons from the various electron donors. The number of protein subunits encoded by nuclear (nDNA) and mitochondrial (mtDNA) genomes are indicated above each complex. Complex I: NADH-dehydrogenase, complex II: succinate-dehydrogenase, complex III: ubiquinone-cytochrome c-reductase, complex IV: cytochrome c-oxidase and complex V: ATP synthase Abbreviations: Cyt c = cytochrome c; Q = ubiquinone; b and c1 = cytochromes; Fe-S = iron-sulfur center; a-Cu and a_3-Cu = the redox-active catalytic cofactors including heme a and copper.

shuttle, malate-aspartate shuttle), and transporters (carnitine palmitoyltransferase II, glutamate aspartate transporter). The other important components are uncoupling protein or glycerol-3-phosphate dehydrogenase, but the most noted is OXPHOS. The system is composed of four respiratory chain complexes and ATP synthase and provide oxidative phosphorylation (figure 2). In this pathway complex I oxidizes nicotinamide dinucleotide (NADH) produced by several catabolic pathways, mainly by the tricarboxylic acid cycle (TCA), fatty acid β-oxidation and the pyruvate dehydrogenase complex. Complex II oxidizes flavine adenine dinucleotide ($FADH_2$) produced by TCA. Complexes I and II transfer electrons from NADH and $FADH_2$ to ubiquinone (coenzyme Q_{10}). Reduced ubiquinone transfers the electrons to complex III, which reduces cytochrome c. Further the electrons are transferred to complex IV and together with molecular oxygen produce water. The transfer of electrons along the respiratory chain (complex I – IV) is used by complexes I, III and IV to pump protons to the intermembrane space generating an energetic protonic gradient. Complex V (ATP synthase), which is not involved in

electron transfer, provides a channel for influx of ejected protons back into mitochondrial matrix. Finally the exergonic protonic flux drives the phosphorylation of ADP to ATP.

Matrix contains soluble enzymes that catalyze TCA cycle, the urea cycle, oxidation of pyruvate and other small organic molecules. The mitochondrial matrix also contains the mitochondrial DNA (mtDNA) and ribosomes.

1.2 The mitochondrial genome in mammalian cells

Human mitochondrial DNA (mtDNA) is a circular double-stranded genome of ~ 16 600 base pairs (figure 3) encoding for 13 proteins of the ~ 90 different proteins present in the OXPHOS (Papa, 1996). The other components of OXPHOS are encoded by nuclear genes and are imported to the mitochondrion via specialized import systems (Chacinska, et al., 2002; Truscott, et al., 2003). The individual strands of the mtDNA molecules are denoted heavy (H) strand and light (L) strand because of their different buoyant densities in a cesium chloride gradient. The observed difference is due to uneven nucleotide content of the two strands, the H strand is guanine rich, whereas the L strand is guanine poor. The mitochondrial genome is very compact due to the lack of intronic or intergenic sequences and the absence of significant 5´ and 3´ untranslated regions (UTRs). However, it is not known why the mitochondrion still retains own genome. There are two main possible explanations. Firstly, mtDNA encodes two highly hydrophobic proteins (COX1 and Cytb) which are difficult to import across the mitochondrial membranes. Second explanation suggests that the regulated expression of mitochondrial genes is important for metabolic control in eukaryotic cells (Allen, 1993). Moreover the differences in codon usage between the nuclear and mitochondrial genomes may make further gene transfer from mitochondrion impossible (Asin-Cayuela and Gustafsson, 2007; Falkenberg, et al., 2007).

About 2-10 mtDNA copies are presented in each of hundreds or thousands of mitochondria per cell and are packaged in a DNA-protein structure called nucleoid (Garrido, et al., 2003; Legros, et al., 2004). The remarkable exceptions are gametes where mature oocyte have approximately $2x10^5$ (May-Panloup, et al., 2007; Reynier, et al., 2001) and sperm about 10^2 (Song and Lewis, 2008) mtDNAs.

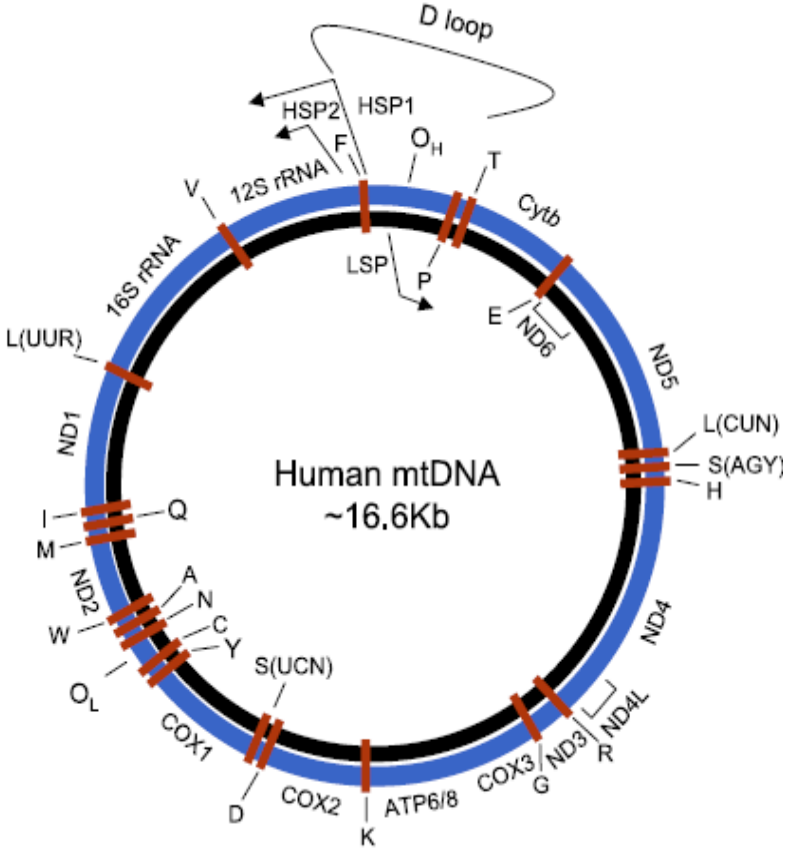

Figure 3. Map of human mitochondrial genome (Scarpulla, 2008a).
Genomic organization of human mtDNA in a circular genomic map showing heavy (blue) and light (black) strands which are named according their behavior in density gradient. Protein coding and rRNA genes are interpolated by 22 tRNA genes (red bars). Duplicate tRNA genes for leucine (L) and serine (S) are distinguished by their codon recognition (parentheses). The D-loop regulatory region contains the L- and H-strand promoters (LSP, HSP1, and HSP2), with arrows showing the direction of transcription. Protein coding genes include the following: cytochrome oxidase (COX) subunits 1, 2, and 3; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5, and 6; ATP synthase (ATPS) subunits 6 and 8; cytochrome *b* (Cyt *b*).

The mammalian mtDNA is maternally inherited and mitochondria in mammalian sperm are destroyed in the fertilized oocyte during the first divisions. This fact affects whole mitochondrial genetics, but there are additional important genetic aspects – heteroplasmy and segregation. When the pathogenic mutation is present in all copies, a situation is called homoplasmy. On the other side, when only a fraction of all copies carries the mutation, it is heteroplasmy. A minimal number of mutant mtDNA is required to manifest a dysfunction of OXPHOS, this is the threshold effect. The threshold is lower in tissues that have high energetic demands (brain, heart, skeletal muscle, kidney and endocrine systems). Segregation of heteroplasmic mtDNA mutations occurs when a cell divides or when mtDNA is regenerated within a postmitotic cell. It explains widely varying levels of mutated mtDNA in different organs (Tesarova, et al., 2004). Studies on mtDNA segregation in pedigrees show that when a point mutation occurs, there may be a complete

switching of mutants to high levels within single generation in maternal lineages (Hauswirth and Laipis, 1982; Poulton, et al., 1998). Taking into account the high mtDNA copy number in mature oocyte and the relatively small number of cell divisions in the development of the female germ line, it has been postulated the bottleneck theory (Hauswirth and Laipis, 1985). According to the theory, the mitochondrial cluster could be extremely reduced before being massively amplified during early oogenesis. This mechanism would tend to eliminate mutated mitochondrial genomes and homogenize mtDNA populations.

1.2.1 DNA replication and transcription in mitochondria

Replication and transcription of mtDNA is completely dependent on nuclear-encoded gene products. MtDNA is replicated by the concerted action of DNA polymerase gamma (catalytic subunit is encoded by *POLG* gene), its accessory subunit p55 (encoded by *POLG2* gene), and replication factors, such as the mitochondrial single-stranded DNA binding protein and the Twinkle helicase. The D-loop, the only mtDNA region for regulation of transcription and replication, which interconnects nucleoid with inner mitochondrial membrane (Albring, et al., 1977), consists of a 1.3 kilobase noncoding region. This region is flanked by the three promoters (H-strand promoters 1 and 2 - HSP1, HSP2 and L-strand promoter LSP) and the transcription termination region for the H-strand. Although basic components of this system have been described, an intense discussion about the mode of mtDNA replication still continues (Brown and Clayton, 2006). The majority of evidence points to mechanism of bi-directional replication where the replication origins for the two strands are displaced by about two-thirds of the genome – the strand displacement model (Brown, et al., 2005; Clayton, 2003). Transcription from the LSP produces the RNA transcript that is cleaved in the proximity of three conserved sequence blocks (CSB I – III) (figure 4). Then the initiation of H-strand replication occurs at the sites of these cleaveges at the origin (O_H). Thus, transcription is coupled to replication and the sites of RNA cleavage are transition sites between RNA and DNA synthesis. A decision must be made to continue transcription through the CSBs or to truncate the nascent RNA to initiate DNA replication. Even after DNA synthesis begins, the nascent strand from the leading H-strand origin is often prematurely terminated at termination associated

sequences (TASs), that is located ~ 700 bp downstream of O_H, giving rise 7S DNA product. This event may be important in controlling mtDNA levels and accounts for the triple-stranded displacement loop structure (D-loop structure) (Falkenberg, et al., 2007; Fernandez-Silva, et al., 2003).

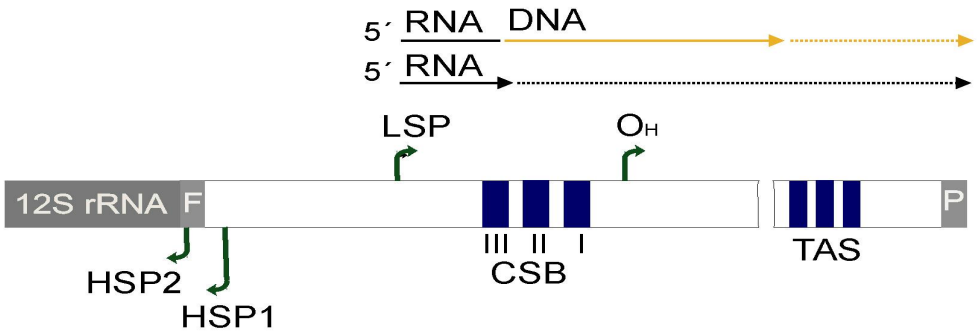

Figure 4. The D-loop regulatory region (redrawn according (Falkenberg, et al., 2007). The three conserved sequence blocks (CSB I, CSB II and CSB III) are located downstream of light-strand promoter (LSP). Transitions from the RNA primer to the newly synthesized DNA have been mapped to sequences within or near CSB II. The conserved termination-associated sequence (TAS) elements are located at the 3´of the nascent D-loop strands and are proposed as a major regulation point of mtDNA replication. HSP = heavy-strand promoter; LSP = light-strand promoter; O_H = origin of H-strand DNA replication, P = proline; F = Phenylalanin

When the leading H-strand synthesis continuous, it exposes the origin of L-strand DNA replication (O_L), which is located at two thirds of the mitochondrial genome. Then lagging L-strand DNA synthesis initiates in the opposite direction. New mtDNA molecules with completed strand synthesis are ligated to form closed circles prior to the introduction of superhelical turns.

POLG is part of a larger mitochondrial replication complex (Graziewicz, et al., 2006; Jazayeri, et al., 2003). POLG is absolutely essential for the organogenesis during mammalian embryonic development (Hance, et al., 2005a). DNA polymerase gamma remains the only DNA polymerase found in mitochondria. Therefore it is responsible for all DNA synthetic reactions including both replication and repair of mtDNA (Kaguni, 2004).

Transcription from the mitochondrial promoters HSP1, HSP2 and LSP produce polycistronic precursor RNAs, encompassing all of the genetic information encoded in each of the specific strands. The HSP2 transcription-initiation site is located close to the 5´end of the *12S rRNA* gene and produces a polycistronic molecule that corresponds to almost the entire H strand, covering two rRNA genes and 12 mRNA-encoding genes. The HSP1 transcription-initiation site is located ~ 100 bp

upstream of HSP2 and produces a transcript that covers two rRNA genes and terminates at the 3´end of the 1*6S rRNA* gene (figure 4). Each of the protein and rRNA genes is flanked by at least one tRNA gene. Excision of tRNA molecules is required to produce mature mRNA and rRNA molecules. This mode of RNA processing is known as the "tRNA punctuation model" (Asin-Cayuela and Gustafsson, 2007; Ojala, et al., 1981). Both HSP and LSP promoters share a critical upstream enhancer that serves as the recognition site for mitochondrial transcription factor A - TFAM (encoded by *TFAM* gene), a member of the high-mobility group (HMG) gene family. The initiation of mtDNA replication depends on TFAM respectively on an RNA primer produced by transcription from LSP (May-Panloup, et al., 2005). TFAM can "bend and unwind" DNA, properties potentially linked to its ability to stimulate transcription upon binding DNA. Moreover to specific promoter recognition, TFAM binds nonspecifically to apparently random sites on mtDNA. This property, along with its abundance in mitochondria, suggests that it plays a role in the stabilization and maintenance of the "mitochondrial chromosome" (Fisher, et al., 1992; Scarpulla, 2008a). In addition to TFAM, mtDNA transcription requires mitochondrial RNA polymerase (POLRMT), specificity factors (TFB1M and TFB2M) and termination factor (MTERF). TFB1M works together with TFAM and POLRMT to direct proper transcription initiation from HSP1, 2 and LSP. The specific function of the two TFB isoforms remains unclear. RNAi knockdown of the Drosophila B2 isoform results in reduced mtDNA transcription and copy number (Matsushima, et al., 2004). Interestingly, the overexpression of either TFB2M or TFAM in this system increases mtDNA copy number whereas overexpression of TFB1M fails to do so. Thus it supports a role for TFAM and TFB2M in mtDNA copy number control (Scarpulla, 2006).

Appropriate OXPHOS subunit stoichiometry requires the coordinate expression of genes on the two genomes and an accounting for a variable number of mitochondrial genomes per cell. This is orchestrated by the nuclear respiratory factors, NRF1 and NRF2 (Ramachandran, et al., 2008).

1.2.2 Nuclear respiratory factor 1 – NRF1

The isolation and characterization of cytochrome c and cytochrome oxidase genes in the early 1980s began a search for transcription factors that are common

to the expression of nuclear respiratory genes (Guarente and Mason, 1983). NRF1 functions as a positive regulator of transcription. NRF1 target genes have been identified by characterization of functional NRF1-binding sites within their promoters. Many NRF1 target genes encode subunits of the five respiratory complexes (Virbasius, et al., 1993). However, the regulatory network controlled by NRF1 extends beyond the respiratory subunits to other classes of genes. These include genes involved in assembly of the respiratory apparatus, constituents of the mtDNA transcription and replication machinery, mitochondrial and cytosolic enzymes of the heme biosynthetic pathway, and components of mitochondrial protein import (Scarpulla, 2006). The fact that *TFAM* gene is an NRF1 target gene consistent with the postulate that NRF1 plays an integrative role in nucleo-mitochondrial interactions. It is likely that the loss of *NRF1* gene affects the expression of NRF1 target genes that are required for cell growth and development (Huo and Scarpulla, 2001; Larsson, et al., 1998).

1.2.3 Nuclear respiratory factor 2 – NRF2

A second nuclear factor designated as NRF2 (also known as GA-binding protein or GABP) is a multi-subunit nuclear transcription factor (Rosmarin, et al., 2004). NRF2 binding sites contained of the GGAA core motif that is characteristic of the ETS-domain family of transcription factors. Human NRF2 includes a DNA-binding α subunit and four others (β_1, β_2, γ_1 and γ_2) that complex with α but alone do not bind DNA. NRF2 β_1 and β_2 contains the transactivating domain and the nuclear localizing signal (LaMarco, et al., 1991; Sawa, et al., 1996). NRF2 is an activator of cytochrome oxidase subunit IV (*COX4* gene) expression (Kelly and Scarpulla, 2004). In addition, a number of other genes related to respiratory chain expression are also target for NRF2 regulation. These include *TFAM*, *TFB1M* and *TFB2M* genes, and subunits of complex II. NRF1 sites are often present in NRF2-dependent promoters, for example in human *TFAM* and *TFB* gene promoters. It is possible that NRF1 may bind sequences that deviate significantly from known consensus (Scarpulla, 2006).

1.3 Mitochondrial biogenesis

Mitochondrial biogenesis can be simply defined as the growth and division of pre-existing mitochondria. It is triggered by enviromental stresses such as exercise, cold exposure, caloric restriction and oxidative stress, cell division and renewal, and tissue differentiation. The complexity of mitochondrial biogenesis involves changes in the expression of more than 1000 genes, the cooperation of two genomes, and alters the level of aproximately 20% of cellular proteins (Lenka, et al., 1998). The genesis of mitochondria includes both mitochondrial proliferation and differentiation processes (Izquierdo, et al., 1995). The proliferation is a long-term program controlled mainly at the transcriptional level of gene expression. Meanwhile the differentiation is a short-term regulation of mitochondrial biogenesis which is controlled at post-transcriptional levels that involve the regulation of the stability and translational efficiency of the mRNAs encoding mitochondrial proteins (Cuezva, et al., 1997).

At the level of the organism, energetic homeostasis is tightly regulated by the hormones. Both thyroid and steroid hormones such as glucocorticoids regulate the expression of most of the nuclear genes encoding mitochondrial proteins. Sex hormones have differential effects on the expression of various mitochondriogenic molecules (Rodriguez-Cuenca, et al., 2007). Other hormones, like adrenal steroids, play an important role in perinatal mitochondrial maturation and biogenesis in a tissue-specific manner (Prieur, et al., 1998). In mammals, the most important factors involved in mitochondrial biogenesis are the thyroid hormones (Mutvei, et al., 1989; Rodríguez-Pena, et al., 2002).

At molecular level, several transcription factors and cofactors are involved in the activation and regulation of mitochondrial biogenesis. These factors can be clustered in the three main groups: ubiquitous transcription factors (Sp1, YY1, CREB (cAMP response element binding), MEF2-box), nuclear respiratory factors (NRF1, NRF2, REBOX/OXBOX-binding factors, MT1 to MT4) and coactivators (PGC1α, PGC1β and PPRC1) (Scarpulla, 2008b). These factors participate in a complex network that also includes hormone-induced signaling pathway components. Despite the complexity of the various signaling pathways that converge to regulate mitochondrial biogenesis, they all seem to share the common key component of the PGC1 family of co-transcription factors. Specifically, PGC1α

has been shown to act as a common intracellular mediator during mitochondrial biogenesis.

1.3.1 PGC1 family - nuclear coactivators in mitochondrial biogenesis

PGC1α (peroxisome proliferator-activated receptor gamma coactivator) is the best-studied member of a family of three related proteins that control major metabolic functions. PGC1α was first identified as a cold-inducible PPAR-γ (peroxisome proliferator-activated receptor gamma) binding protein in brown fat (Puigserver, et al., 1998; Scarpulla, 2008b). This coactivator lacks DNA-binding activity but interacts with and coactivates numerous transcription factors including NRFs on the promoter of *TFAM* gene. Thus, PGC1α may link nuclear regulatory events to the mitochondrial transcriptional machinery (figure 5). Indeed, PGC1α seems to be a crucial factor in both the activation of the full program of mitochondrial biogenesis and in respiration. Its physiological importance is underscored by the fact that expression levels of PGC1α are directly related to mitochondrial biogenesis activity. Therefore it offers promising venue for treatment of mitochondrial disorders (Cui, et al., 2006; Wenz, 2009). Ectopic expression of *PGC1α* gene in cultured myoblasts and other cells induces respiratory subunit mRNAs and increases COX4 and cytochrome c protein levels as well as the mtDNA level (Wu, et al., 1999). In addition, *PGC1α* mRNA level correlates with skeletal muscle oxidative capacity, suggesting that it plays a major role in setting mtDNA content (Garnier, et al., 2003). *PGC1α* gene expression is enhanced in the developing mouse heart immediately before the burst of mitochondrial biogenesis that precedes birth (Lehman, et al., 2000). During the neonatal stages, inducible cardiospecific overexpression of *PGC1α* gene leads to a dramatic increase in mitochondrial number and size, concomitant with upregulation of genes associated with mitochondrial biogenesis (Russell, et al., 2004; Ventura-Clapier, et al., 2008).

PGC1α has two structural homologs, PGC1β and the more distant PGC1-related coactivator (PPRC1), which have been less extensively studied. PGC1β shares sequence similarity with PGC1α along its entire length (Lin, et al., 2002). Tissue mRNA expression level of PGC1β parallels with *PGC1α* mRNA level with

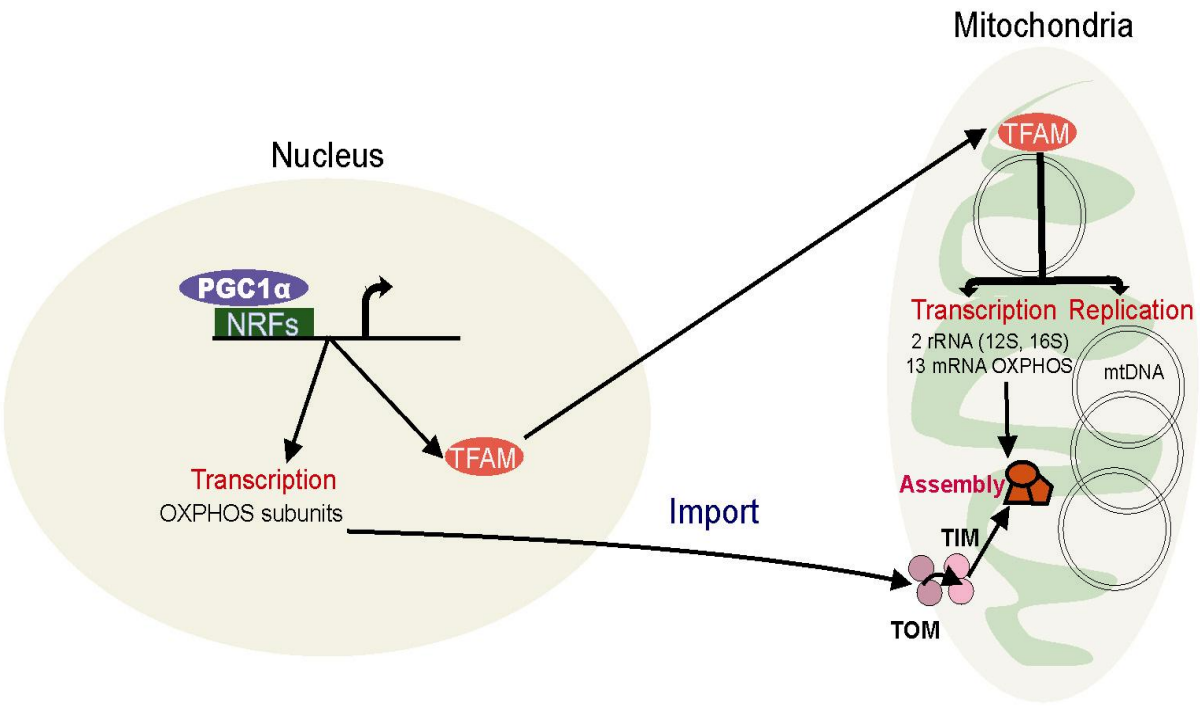

Figure 5. Schematic representation of PGC1α stimulation of mitochondrial transcription and replication (redrawn according (Ventura-Clapier, et al., 2008).
PGC1α activates nuclear respiratory factors (NRFs) leading to transcription of nuclear-encoded OXPHOS proteins and of the mitochondrial transcription factor TFAM. TFAM activates transcription and replication of the mitochondrial genome. Nuclear-encoded subunits are imported into mitochondria through the outer (TOM) or inner (TIM) membrane transport machinery. Nuclear and mitochondrial encoded subunits of the respiratory chain are then assembled.

the highest levels in brown fat, heart, brain, liver and skeletal muscle, tissues high in mitochondrial content and oxidative energy production. PGC1β also binds NRF1 and is a potent coactivator of NRF1 target genes (St-Pierre, et al., 2003). However, PGC1β differs from PGC1α in that it is not induced in brown fat upon cold exposure (Meirhaeghe, et al., 2003) and preferentially induces genes involved in the removal of reactive oxygen species (St-Pierre, et al., 2003). Overexpression of *PGC1β* gene in cultured muscle cells result in increased mitochondrial biogenesis and oxygen consumption (Lin, et al., 2002). Thus, although PGC1α and PGC1β are probably functionally distinct, they both utilize NRF1 and other transcription factors to induce mitochondrial biogenesis.

In contrast to *PGC1α* gene, *PPRC1* gene is largely ubiquitously expressed, is only slightly induced in response to cold exposure, and is cell-cycle-regulated (Andersson and Scarpulla, 2001). However, PPRC1 may be capable of regulating mitochondrial function in a manner similar to PGC1α. PPRC1 activates the

transcription of NRF1 target, cytochrome c, but requires the cooperation of other factors including CREB (Andersson and Scarpulla, 2001). In addition, *PPRC1* gene expression is not particularly enriched in highly oxidative tissues with abundant mitochondria. *PPRC1* gene is induced upon serum treatment of quiescent fibroblasts and is expressed more abundantly in proliferating cells compared with growth-arrested cells (Gleyzer, et al., 2005). Thus, PPRC1 may link the expression of genes required for both cell growth and mitochondrial respiration (Scarpulla, 2008a).

1.3.2 The role of thyroid hormones in mitochondrial biogenesis

Triiodothyronine (T3), the active form of thyroid hormone (TH), has a profound influence on normal development, differentiation and metabolism (e.g. T3 regulates mitochondrial biogenesis and metabolism in heart during maturation) (Portman, et al., 2000; Yen, 2001). Injection of T3 in whole animals or T3 therapy in man increases oxygen consumption, metabolic rate, body temperature and heart rate. This is followed by a rapid meltdown of adipose tissue and muscle wasting (Tata, et al., 1963). T3 mainly affects the energy metabolism in heart, muscle and liver. Thyroid hormone induces multiple effects through non-genomic and genomic modes of action. The molecular mechanism of the non-genomic actions are incompletely understood (Davis, et al., 2002). The genomic effects include the regulation of gene expression by way of thyroid hormone receptors (TRs). TRs belong to a large family of ligand-dependent transcription factors. T3 serves as the primary ligand for these receptors. Thyroid-stimulated mitochondrial biogenesis is probably mediated via specific TRs located in both the nuclear and mitochondrial compartments which trigger a series of transcriptional and cell signaling events leading to a greater content of mitochondria (Wrutniak-Cabello, et al., 2001). Some observations identified two truncated isoforms of TRα1 (generated by alternative translational initiation at internal start codons) exclusively in the mitochondria (Wrutniak, et al., 1995).

TRs bind to thyroid hormone response elements (TREs), which typically consists of two half-sites containing the sequence 5´- AGGTCA -3´ in various orientations, separated by a spacer of various length (Yen, 2001). Ligand binding promotes recruitment of coactivators, which modulate transcriptional activity of genes

involved in oxidative phosphorylation or substrate oxidation (Jansen, et al., 2000; McClure, et al., 2005; Miyamoto, et al., 1997). However, T3 also regulates genes containing no apparent TRE within the promoter region (Weitzel, et al., 2003). This fact supports metabolic regulation by T3 through intermediate factors, including transcriptional activators and coactivators. Thus TH working through direct and indirect modulation of oxidative phosphorylation and substrate oxidation genes could coordinate developmental modifications in energy-producing pathways during prenatal development. Evidence for this coordination is provided by the prenatal TH surge, which coincides with a switch in myocardial substrate preference from carbohydrate in fetal life to fatty acids shortly after birth (Breall, et al., 1984; McClure, et al., 2005).

One of the major targets of T3 is the modulation of mitochondrial biogenesis and activity. Therefore common DNA motifs in gene promoters of nuclear-encoded mitochondrial proteins has been searched. Indeed, some common DNA binding motifs have been identified in these promoters serving as candidates to participate in a coordinated response to T3. They include the OXBOX/REBOX and the Mt motifs, and the recognition sites for the regulatory proteins Sp1, YY1, NRF1 and NRF2. Studies in T3-treated rats identified *NRF1* and *NRF2* genes to be regulated by this hormone (Rodríguez-Pena, et al., 2002). The functional NRF1 binding sites have been characterized in T3-induced genes, e.g. cytochrome c and *TFAM*. The mitochondrial as well as the nuclear genome appear to be regulated in a similar manner in response to T3 (Weitzel, et al., 2003).

1.3.3 Mitochondrial biogenesis during prenatal development

The efficient mitochondrial biogenesis is considered as an important factor for both successful maturation of oocyte and postnatal adaptation to extrauterine life (May-Panloup, et al., 2007). Therefore the inadequate efficiency of mitochondrial biogenesis leads to low energy production which may play a crucial role both in the fetal development and neonatal morbidity. The complete understanding of the regulation of mitochondrial function and biogenesis may improve the care of premature neonates. Moreover it could be benefit for diagnostics of the mitochondrial disorders with neonatal onset in critically ill neonates (Arnon, et al.,

2002; Honzik, et al., 2008). There are several stages where the mitochondrial biogenesis is the critical factor for onward cell or tissue development – the oocyte maturation and selection, the placentation, the early postnatal adaptation.

Sufficient activation of mitochondrial biogenesis is necessary for selection of the oocyte during maturation. Oocyte growth and maturation may be improve by addition of several hundreds of mitochondria (May-Panloup, et al., 2007; Perez, et al., 2000).

In the oocyte, the stage of mitochondrial biogenesis may be reflecting by the mtDNA content. According some studies, the mtDNA content is highly variable in human oocytes (Reynier, et al., 2001; Steuerwald, et al., 2000) and it could be explained by differences in maturation levels of the oocytes (May-Panloup, et al., 2007).

The mitochondrial biogenesis also takes place during the placentation period (Alcolea, et al., 2006). On gestational day 12, the rat placentary circulation is established and oxygen becomes more available to the embryo, the mitochondria undergo considerable morphofunctional changes coinciding with the partial switch from glycolytic to oxidative metabolism (Alcolea, et al., 2006; Shepard, et al., 1998; Shepard, et al., 1997). In this way rat embryo mitochondria reaches a more differentiated stage with a more efficient oxidative metabolism that facilitates the embryo growth during the second half of the gestation (Alcolea, et al., 2007). Similarly in human and primates, mitochondria pass through ultrastructural changes with initiation of mitochondrial biogenesis during early embryo development (Enders and Schlafke, 1981; Sako, 1975; Shepard, et al., 1998).

Finally, at birth, the adaptation to extrauterine environment involves profound hormonal, physiological and metabolic changes (Pollak, 1977; Sutton and Pollak, 1980; Valcarce, et al., 1994; Valcarce, et al., 1988). Indeed the full switch from glycolysis to respiration relies on the mitochondrial biogenesis which is already initiated in oocyte selection during maturation and then during the placentation period.

1.3.3.1 The changes of mtDNA amount

The detailed data about the physiological changes of mtDNA amount in later fetal development are still scarce. There are studies focused on changes of mtDNA amount during fetal development in both human and animal, but most of them are concerned with the mtDNA changes in very early fetal development (Alcolea, et al., 2006; Garcia, et al., 2000; May-Panloup, et al., 2005; Reynier, et al., 2001; Steuerwald, et al., 2000; Tamassia, et al., 2004). The better knowledge of these changes in later fetal development could elucidate a mechanism of rapid adaptation to respiratory demands of a cell during postnatal switch of glycolytic to oxidative metabolism. The mtDNA level changes are often accompanied by mitochondrial gene expression changes (Papa, 1996). Thus, the increase or the decrease in mitochondrial genomes may reflect a response to respiratory demands of the developing tissues. The imbalance of mtDNA level could have an impact on energy availability in developing tissues and therefore potentially also on secondary postnatal morbidity (Honzik, et al., 2008).

There are several studies focused on changes in mtDNA amount in various tissues during aging. The study of Heerdt and Augenlicht (1990) described no significant mtDNA content changes in human cord blood leukocytes (HCBL) during the second part of gestation. On the contrary Pejznochova et al. (2008) observed significant decrease of mtDNA content in HCBL during this period. Gadaleta et al. (1992) described the increase of mtDNA copy number with aging in liver, heart and brain between adult and senescent rats and Nicklas et al. (2004) showed that the relative mtDNA copy number in rat liver rises at a young age (3-60 days), then decreases and holds fairly steady to 2 years of age. Morten et al. (2007) and Pejznochova et al. (2010) found out that the mtDNA content in human fetal liver increases during development. Barrientos et al. (1997a, 1997b) analyzed the changes of mtDNA amount during aging in human brain and skeletal muscle and found out that the mtDNA content increased significantly with age. Also Garcia et al. (2000) found an increase of mtDNA content during early human development in heart. On the contrary, Miller et al. (2003) demonstrated no significant change in mtDNA copy number with age in human skeletal muscle and heart. The interesting difference is among some studies in method used for the mtDNA quantification.

Miller et al. (2003) used the real-time PCR method but Barrientos et al. (1997a, 1997b) applied Southern blot method and Garcia et al. (2000) performed the mtDNA quantitation by the quantitative competitive PCR assay. According to Chabi et al. (2003) and Bhat and Epelboym (2004), the real-time PCR is more accurate and reproducible method for mtDNA quantification than other methods.

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1.3.3.2 Fetal liver and hematopoiesis

Development during the fetal period is concerned with differentiation of tissues, organs and systems and at the cell level with mitochondriogenesis (Persaud and Moore, 1998).

In the human fetus, the liver are formed from two of the three basic embryonic tissues, the mesoderm and the endoderm, early in the 4th week of gestation (Jones and Rolph, 1985). The fetal liver may to form up to 10% of fetus weight (Masopust, 2003). Its development occurs rapidly, with the basic liver elements and structure formed by the end of the first trimester of gestation. Initially, in the septum transversum, endodermal cells differentiate into bipotential hepatoblasts that can can differentiate into either hepatocytes or biliary epithelial cells. During the 5th to 6th week of development, hepatoblasts acquire a biliary phenotype and form layer rimming the portal tract. The tract becomes later a double layer and subsequently develops a central lumen of the ductal plate. Hepatocytes develop the adult morphology about 5th year of life (Ring, et al., 1999; Russo, 2007).

Over prenatal period, fetal liver develops the ability to synthesize large amounts of protein, then fat, followed by glycogen and glucose. The synthesis of fetal glycogen starts in the 9th week of gestation, but the production is peaking shortly before birth, when the fetal liver contains 2-fold higher amount of glycogen then adult liver. Glycogenolysis and following glycolysis are the main source of metabolic energy immediatelly after birth (Masopust, 2003).

Fetal liver has a greater cellular heterogeneity than in adult. There are numerous hematopoietic cells among hepatoblasts. Fetal hematopoiesis moves through several overlapping anatomic and functional stages, beginning in the yolk sac and entering in the hepatic phase at 6th week of gestation and the marrow phase at 20th week of gestation (Figure 6). The progression of hematopoiesis in the fetal liver

reaches its maximum around 12th week of gestation and then continuously decreases and simultaneously increases in the bone marrow till birth (Brugnara and Platt, 2003). The shift from fetal liver to bone marrow is associated with marked alterations in functional properties of blood cells and the fetal liver continues to produce formed elements of blood into first postnatal week. It means that the blood cells are produced simultaneously from the fetal liver and the bone marrow in the period from 20th week of gestation to the first postnatal week. Transfer to the bone marrow phase is generally complete at birth (Tavian and Péault, 2005). Through this change the proliferation of the hepatocytes is rising simultaneously with mitochondrial proliferation, consequently the mtDNA content increases (Izquierdo, et al., 1995).

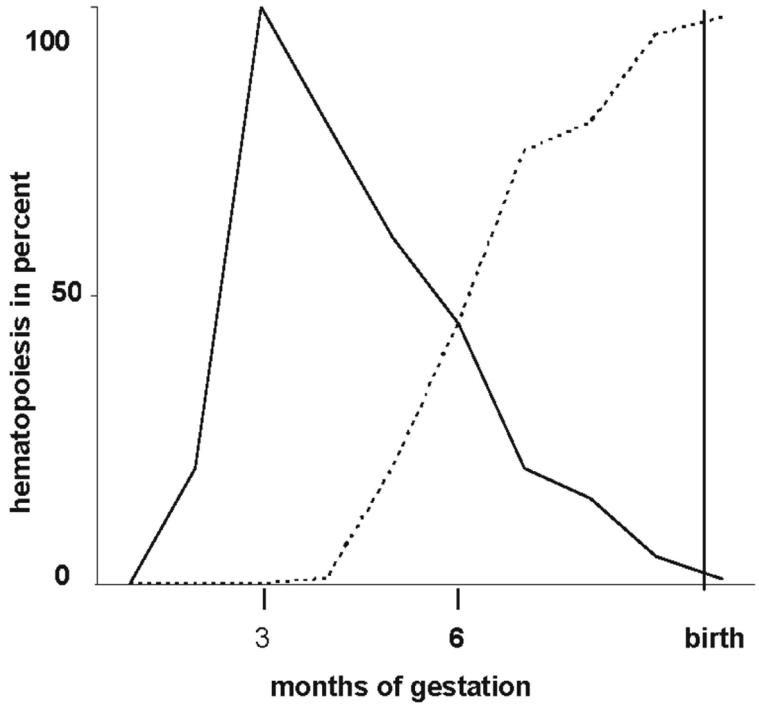

Figure 6. The change of hematopoietic site during prenatal development (redrawn according Brugnara and Platt, 2003). The solid line shows fetal liver hematopoiesis and dashed line shows bone marrow.

1.3.3.3 Fetal muscle

Through gestation the skeletal muscle of fetus changes from a poorly differentiated collection of myotubes to well-ordered striated myofibres with good glycolytic and contractile capacity. Skeletal muscle arises from the mesoderm (Jones and Rolph, 1985).

Maturation of skeletal muscle compared with cardiac muscle presumably reflects the differences in the energetic demand between them in utero. Largely undifferentiated myoblasts which have afoul developed contractile apparatus, fuse to form myotubes. These consist predominantly of sarcoplasm with limited myofilaments. Myoblasts develop into myofibres with dominant myofilaments. Early myofibres lack of metabolic differentiation. These fibers have weak activities of both glycolytic and oxidative enzymes (Jones and Rolph, 1985). Consequently they differentiate into three basic adult fiber types : type I (slow-twitch, with high oxidative capacity and low activities of glycolytic enzymes), type IIA (fast-twitch oxidative glycolytic, with high activities of glycolytic and oxidative enzymes) and type IIB (fast-twitch glycolytic, which is similar to IIA except for oxidative capacity that is ussually low).

The terminally differentiated cells of human skeletal muscle are formed in three distinct waves. The first wave proceeds in embryonic period. The secondary muscle fibres begin to form in fetal period (from 9^{th} week of gestation to birth) and the tertiary wave of myofibre formation continues past the time of birth (Miller, 2001; Persaud and Moore, 1998). Between the first and the secondary waves, the mtDNA amount shows no changes (Minai, et al., 2008). On the other hand muscle differentiation is associated with the increase of mtDNA content during rat embryonic development (Franko, 2008; Miki, et al., 1988). It is supported by the fact that glycolytic energy production in proliferating myoblasts shifts to oxidative phosphorylation in differentiated myotubes (Franko, 2008; Leary, et al., 1998). This process proceeds probably since the secondary waves (Pejznochova, et al., 2010).

1.3.4 Adaptation to extrauterine life

Succesful transition to extrauterine life requires that the fetus, previously poikilothermic, with the placenta subserving most of its metabolic needs, becomes homeothermic and self-sustaining. The function of gas exchange is transferred from the placenta to the lungs and this is accomplished by elimination of the umbilical-placental circulation, increase in pulmonary blood flow and closure of the ductus arteriosus and foramen ovale (Breall, et al., 1984). In human, this change is represented by the transition from hypoxic fetal environment with partial oxygen pressure 18 mm Hg (in amniotic fluid between 11 and 16 weeks of gestation) (Jauniaux, et al., 2001) to oxygen-rich environment where the partial oxygen pressure in blood of neonate reaches about 50 mm Hg after 96 hours (Persaud and Moore, 1998). The coordinated actions of adrenal cortical, adrenal medullary and thyroid hormones facilitate the transition to air breathing, neonatal cardiovascular adaptation, glucose homeostasis, thermogenesis and gut maturation (Fisher, 2008).

In most mammals, extrauterine adaptation is initiated by a cortisol surge. Cortisol is a corticosteroid hormone or glucocorticoid produced by zona fasciculata of the adrenal cortex, which is a part of the adrenal gland. It is usually referred to as the "stress hormone". The increase in fetal circulating cortisol concentration during the final weeks of gestation is associated with a variety of physiologic responses (Gluckman, et al., 1999; Wallace, et al., 1995). These include increased conversion T4 to T3 in liver; increased adrenaline secretion; augmented synthesis and secretion of surfactant and maturation of surfactant composition; stimulation of maturation of hepatic gluconeogenic enzyme activities; induction and maturation of a variety of gut enzymes for nutrient absorption and maturation of gut transport processes, motility and structure (Fisher, 2008).

Parturition and umbilical cord cutting evokes a dramatic catecholamine surge in the newborn, resulting in extraordinarily high serum levels of norepinephrine (noradrenaline), adrenaline and dopamine (Padbury, et al., 1981). These changes evoke critical cardiovascular adaptations including increased blood pressure and increased cardiac inotropic effects, increased glucagon secretion, decreased insulin secretion, increased thermogenesis in brown adipose tissue and increased free

fatty acid levels and pulmonary adaptation including absorbtion of pulmonary fluid and increased surfactant release (Gluckman, et al., 1999).

In addition to the cortisol and catecholamine surge, parturition stimulates a postnatal TSH surge (thyroid-stimulating hormone or thyrotropin) that peaks at 70 to 100 mU/L within first hour after birth. The surge is triggered by umbilical cord cutting and cooling of the newborn in the extrauterine environment. The TSH surge stimulates newborn thyroid gland T4 and T3 secretion (de Zegher, et al., 1994; Fisher, 2008).

1.3.4.1 Maturation of thyroid system

THs which have main effect on maturation of fetus are produced by thyroid system. Its ontogenesis can be divided into five phases: embryogenesis, hypothalamic-pituitary-thyroid system (HPT) maturation, thyroid hormone metabolism, ontogenesis of thyroid hormone effects and maturation of thyroid system control. By 12 weeks of gestation thyroid system is largely complete, including the hypothalamus, pituitary and thyroid gland (Fisher, et al., 1976). However, thyroxine secretion is limited until midgestation when the fetal HPT axis begins to function. HPT maturation continuous through 35 to 40 weeks of gestation, during which time there is a progressive increase in hypothalamic thyrotropin releasing hormone (TRH) and maturation of the hypothalamic-pituitary portal vascular system (Burrow, et al., 1994). The fetus progresses from a state of both pituitary and hypothalamic thyroid system immaturity at midgestation through a state of forward hypothalamic maturation during the final weeks of pregnancy. Premature infants at less than 28 weeks of gestation respond to exogenous TRH with an increase in TSH concentration comparable to adults. However, their TSH response to extrauterine cooling is subdued, indicating hypothalamic immaturity (Fisher, 2008; Murphy, et al., 2004). It could be also reason why the premature infants have inefficient capacity of OXPHOS.

1.3.4.2 OXPHOS

At the end of the fetal life, the mitochondrial oxidative capacities are low, but reach 80% of the adult values one day after birth (Cuezva, et al., 1997). The switch alone from fetal anaerobic glycolysis to neonatal OXPHOS is extremely rapid process. Within one hour after birth, an activation of mitochondrial protein translation occurs in the liver (Valcarce, et al., 1988). The changes in the physiology of hepatocyte mitochondria at birth play a part in lowering the lactate/pyruvate ratio from 213 in the newly born rat to 13.5 within 1h after birth (Philippidis and Ballard, 1969). By 12h after birth the glycogen reserves are totally depleted (Chiu and Phillips, 1974). The activation of glycogenolysis and glycolysis as a result of the cyclic AMP-mediated actions of adrenaline and glucagon results in an increase in the cytoplasmatic ATP concentration, which causes the hypercontraction of the mitochondrial inner membrane, leading to mitochondrial maturation (Sutton and Pollak, 1980).

The increase in the rate of synthesis of mitochondrial protein is accomplished by prenatal accumulation of nuclearly and mitochondrially encoded mRNAs (Luis, et al., 1993), which are efficiently translated immediately after birth. The increase in intramitochondrial adenine nucleotide concentration promotes coupling efficiency between respiration and oxidative phosphorylation (Pollak, 1977).

It is also known that the change of partial oxygen pressure has impact on OXPHOS activation (Shepard, et al., 1998; Shepard, et al., 1997). There could be differences in accessibility of oxygen among tissues immediately after birth and therefore the activation of OXPHOS might be tissue-specific. This process is well described in switch of COX isoforms. The switch of Cox6aL isoform (liver – non-muscle specific) to Cox6aH isoform (heart/muscle specific). In the mouse early embryo, COX VIa-L is the predominant isoform expressed in all tissues. At late fetal stages up until birth, COX VIa-L and -H are both expressed in myocytes, but the L isoform remains the dominant form. In postnatal striated muscle, expression of the H form increases whereas L decreases in a reciprocal manner (Kim, et al., 1995; Parsons, et al., 1996). The activation of COX in muscle tissue is started by higher partial oxygen pressure which probably leads to the change of isoforms. In liver the activation of COX is not associated with the isoform switch. The overall postnatal changes in liver are gradual compared to muscle. It is affected by the fact that

maturation of liver tissue has to proceed earlier than maturation of skeletal muscle to provide postnatal metabolism and homeostasis. It could also be the reason of the tissue-specific changes in mtDNA content and OXPHOS gene expression during human development (Heerdt and Augenlicht, 1990; Pejznochova, et al., 2010).

These three processes – the efficient translation due to accumulation of mRNAs, the increase in intramitochondrial adenine nucleotid concentration and the change of partial oxygen pressure – are main in postnatal onset of OXPHOS.

2 AIMS OF THE STUDY

Human prenatal development is a period of great interest in the field of mitochondrial biogenesis since its adequate capacity plays an important role in postnatal adaptation to extrauterine life. The process of OXPHOS maturation during the development of the fetus is still not fully understood. The better understanding of the regulation of mitochondrial biogenesis may improve the care of very premature neonates, especially critically ill premature neonates.

The thesis is based experimentally on molecular genetic analyses in human cord blood leukocytes (HCBL) and placental tissue obtained after the delivery of neonates in various gestational age and samples of fetal muscle and liver tissues obtained at autopsies after termination of pregnancy for genetic indications unrelated to primary OXPHOS disorders. The most important requirement for successful molecular genetic analyses in the study was satisfying RNA quality in fetal tissue samples.

The specific aims of this study have been:

a) to determine appropriate conditions for handling and storage of fetal tissue samples with a focus on effect on analysis of the mRNA expression by quantitative real-time PCR method.
b) to analyze the changes in the mtDNA content in human cord blood leukocytes (HCBL) during human fetal development between 25th and 41st week of gestation and in human fetal liver and muscle tissues between 13th and 28th week of gestation.
c) to characterize the trends in expression of the genes involved in mtDNA transcription, regulation and maintenance (*POLG, TFAM, NRF1, NRF2, PGC1A, PGC1B* and *PPRC1* genes) and genes encoding OXPHOS subunits (nuclearly encoded genes – *COX4, ATP50, ATP5G2* and mitochondrially encoded genes – *MTCO2, ATP6*) in human fetal liver and muscle tissues between 13th and 28th week of gestation.

3 MATERIAL AND METHODS

3.1 Fetal part of placental tissue for evaluation conditions for handling and storage of fetal tissue samples

Samples of placental tissue were obtained from fetal part of placenta after informed consent from 20 term neonates (10 boys and 10 girls) with birth weight 3477 ± 573 g (range 2610 – 4730 g) and gestational age 40 ± 1 weeks (range 37 – 42 weeks) (Table 1). The tissue samples were frozen immediately after withdrawal of placenta and washing it by physiological buffer, 3 – 6 samples were frozen according to the time-plan (1.interval = 1 hour, 2.interval = 2 hours and 3.interval = 3 hours) for time-course study (the part of them was kept in 0°C and the other part was kept in 24°C). Nine neonates were delivered by the Caesarean section, the other eleven vaginally. In five neonates early postnatal adaptation was complicated because of mild perinatal asfyxia with low Apgar score (3-7 in the fifth and tenth minutes).

3.2 Human cord blood samples for DNA isolation from leukocytes

Cord blood samples were collected from the placental part of the umbilical cord immediately after the delivery of the child (n = 107). Neonates (47 boys and 60 girls) were born at the gestational age between 25 – 41 weeks of gestation and all pregnancies were uneventful till birth (Table 2). Family history in all neonates were without any symptoms of mitochondrial disorders. The group of 107 cord blood samples was divided into 13 subgroups according to the week of gestation.

3.3 Fetal liver and muscle tissues

Twenty-six pairs of human fetal liver and muscle tissues were obtained at autopsy after termination of pregnancy for genetic indications unrelated to OXPHOS deficiency between 13^{th} and 28^{th} week of gestation. In all cases there was no family history of mitochondrial disorders. Tissues were immediately immersed in liquid nitrogen and stored at -80°C until analysis. The mean fetal weight was 334 ± 269 g (range 55 – 1200 g). Main clinical data in 26 terminations of pregnancy are shown in Table 3. Various reasons were indication for terminating of the pregnancy like

trisomy 21 (10 fetuses), trisomy 18 (1 fetus), sacral teratoma (2 fetuses), Occipital meningocele (2 fetuses), oligohydramnion (2 fetuses), spina bifida (2 fetuses) and others congenital defects.

3.4 Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics at the General University Hospital in Prague. Informed consents were obtained from women prior to taking tissue samples. All control samples were obtained after informed consent of the parents.

Table 1. Gestational age and birth weight of 20 neonates whose placenta was used in the study.

Patient No.	Gestational age (week)	Birth weight (g)	Gender male / female
1	39+6	3900	m
2	39+5	3520	f
3	40+3	4010	f
4	40+6	4730	m
5	40+1	3190	m
6	38+1	3000	m
7	42+2	2610	f
8	38+3	3210	f
9	38+6	3140	m
10	40+5	3740	m
11	37+5	2750	f
12	39+3	3680	m
13	42+3	3390	f
14	41+4	3820	m
15	39+2	2780	m
16	39+0	3620	m
17	38+4	2720	f
18	40+4	3800	f
19	39+4	3440	f
20	40+2	4480	f

Table 2. Gestational age and birth weight of 107 newborns whose cord blood was used in the study.

Subgroup	Number (n)	Gestational age (week)	Birth weight (g) median (range)	Gender (n) male / female
1	3	25	799 (660 – 980)	1 / 2
2	4	28	1220 (1070 – 1360)	2 / 2
3	8	30	1663 (1270 – 2500)	4 / 4
4	4	32	1388 (1150 – 1625)	0 / 4
5	11	33	2068 (1760 – 2545)	5 / 6
6	7	34	2485 (1690 – 3580)	6 / 2
7	13	35	2215 (1740 – 2640)	10 / 3
8	6	36	2543 (1570 – 3000)	2 / 4
9	8	37	2957 (2650 – 3460)	5 / 3
10	8	38	3314 (2710 – 5060)	1 / 6
11	13	39	3540 (3090 – 3900)	5 / 8
12	11	40	3286 (2790 – 3830)	2 / 9
13	11	41	3751 (3350 – 4000)	4 / 7

Table 3. Clinical data in 26 terminations of pregnancy.

Patient No.	Gestational age (week)	Fetal weight (g)	Main clinical complication
1	13+0	85	hygroma colli cysticum
2	13+5	70	trisomy 21
3	13+6	55	trisomy 21
4	14+2	75	trisomy 21
5	16+0	90	trisomy 21
6	16+4	85	trisomy 18
7	16+4	110	trisomy 21
8	17+4	100	spina bifida
9	18+0	350	gastroschisis
10	18+0	345	occipital meningocele
11	19+0	440	abortus spontaneus
12	19+1	300	trisomy 21
13	19+4	280	trisomy 21
14	20+0	290	trisomy 21
15	20+0	360	congenital defect
16	20+1	300	sacral teratoma
17	20+3	280	deformity of limbs
18	20+3	355	trisomy 21
19	20+5	150	omphalocele
20	21+3	510	spina bifida and meningocele
21	22+0	350	agenesis of kidney and oligohydramnion
22	22+1	400	oligohydramnion,hypotrofy
23	23+2	600	trisomy 21
24	23+5	610	congenital defect
25	25+4	1200	sacral teratoma
26	28+3	900	occipital meningocele

3.5 DNA and RNA isolation from tissues

DNA and RNA were always isolated simultaneously from liver and muscle fetal tissue samples by TriReagent solution (MRC). The placenta tissue was used to RNA isolation by the same commercial solution. All the isolation processes were performed according to the recommended manufacturer protocols.

DNA was stored at 4°C. Total RNA was treated by DNase I (Ambion) according to manufacturer protocol. The quality of total RNA was checked by Agilent Bioanalyzer 2100 (Agilent Technologies) and NanoDrop 1000 (Thermo Scientific).

3.6 DNA isolation from cord blood

All cord blood samples were taken into tubes with EDTA (BD Vacutainer ®). DNA was extracted from HCBL by the isolation according Kendall et al. (1991) with some modifications. For selective lysis of erythrocytes was used modified lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 50 mM EDTA, pH 8) and lysate was digested by proteinase K in SDS and EDTA (10% SDS, 0.5 M EDTA). The extracted genomic DNA was diluted in TE buffer and stored at 4°C.

3.7 Reverse transcription

One thousand nanograms of total RNA was transcribed to cDNA using Superscript III Reverse Transcriptase (Invitrogen) and Oligo-dT primers (Promega) (thermal conditions - Table 4). RT-minus controls were always prepared (no reverse transcriptase added). cDNA was stored at -20°C until analysis by real-time PCR, but at most for two weeks.

3.8 RNA electrophoresis and RIN

The electrophoresis was used to check RNA integrity and purity and was prepared according Masek et al. (2005). For placenta tissue samples was used analysis of RNA integrity number.

The RNA integrity number (RIN) is a software tool designed to help estimate the integrity of total RNA samples. Using this tool, sample integrity is not determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA

sample. This includes the presence or absence of degradation products (Agilent Technologies - www.home.agilent.com).

The fragments of *MECP2* gene - 378bp fragment which involves the exon-exon boundaries and 1134bp fragment which includes intron - were applied to control of genomic DNA (gDNA) contamination in placenta RNA respectively cDNA samples (in case of the contamination, there were found both fragments) (Table 5).

Table 4. Reverse transcription – reaction mixture and thermal cycling conditions.

mixture 1 (10 µl)	**final concentration in 1 reaction (20 µl)**	**Thermal cycling conditions**		
		Step	**Temperature**	**Time**
RNA template	1-2 ug	1. initial denaturation	72°C	2´
Oligo dT primer	0.5 ug			
mixture 2 (10 µl)	**final concentration in 1 reaction (20 µl)**			
FSB	1x			
DTT	10 mM	2. reverse transcription	42°C	50´
dNTPs	0.5 mM	3. inactivation of enzyme	70°C	15´
RNAsin	20 U			
SuperScript III	100 U			

Table 5. PCR reaction mixture and thermal cycling conditions for *MECP2* fragment – control of gDNA contamination.

PCR reaction mixture	**final concentration in 1 reaction (25 µl)**	**PCR conditions**		
		Step	**Temperature**	**Time**
		1. initial denaturation	95°C	2´
DNA template	50-100 ng	2. denaturation	95°C	30´´
CombiPPPMaster Mix	500 µg / ml	3. annealing	60°C	30´´
primer forward	1x	4. extension	72°C	1´
primer reverse	10 mM	30 times repeating steps 2 to 4		
		5. final extension	72°C	10´

3.9 Real-time PCR

Real-time PCR amplification was performed in 20 µl containing 2X Master Mix DyNAmo^{TM} HS SYBR® Green (Finnzymes), 200 nM each primer and 1µl of DNA or cDNA template. The concentration of each analysed DNA sample was 50 ng/µl and each cDNA sample was diluted in a way that 1 µl of cDNA correspond to 25 ng of total RNA used for reverse transcription. The thermal cycling conditions were 95°C

for 15 min, 40 cycles of 95°C for 15 s and target specific annealing temperature and time (Table 6) and then 72°C for 30 s. The following steps were : 72°C for 5 min, the melting curve from 72°C to 92°C (fluorescence reading every 1°C / 10 s) and 72°C for 10 min. Data were collected using Chromo4 real-time PCR system (Bio-Rad).

Some genes were analysed by 7300 Real-Time PCR System (Applied Biosystems). TaqMan Gene Expression Assays (Applied Biosystems) were applied to analyse gene expression of *NRF2* (Hs01022023_m1), *PGC1B* (Hs00993804_m1) and *PPRC1* (Hs01563930_m1). The thermal cycling conditions and reaction mixtures were prepared according to manufacturer protocols. Other Real-time PCR primers are shown in table 6.

Ten-fold serial dilutions of the genomic DNA (from 100 ng to 10 ng) or two-fold serial dilutions of the cDNA (from 50 ng to 3 ng of total RNA which was transcribed to cDNA) were included in each run to generate the calibration curve. All samples were analysed twice in triplicate for each gene. At least one no-template-control or RT-minus control was included within each experiment. A calibration curve was rejected if the correlation coefficient of the trend line was < 0.99. Occasionally, one point from particular triplet was rejected if it deviated significantly from the trend line. The slope of the calibration curve reflected the reaction efficiency. Analysis of reaction specificity was provided by melting curve analysis and electrophoresis.

3.10 mtDNA analysis

To quantify the mtDNA content, we selected two genes, namely, *MT-RNR2* as a mitochondrial target and *GAPDH* as a nuclear target (Table 6). The nuclear target was used to quantify nuclear DNA and therefore normalization of the mtDNA amount per cell was accomplished. The equation (1) was applied to express the mtDNA amount as mtDNA level per cell.

$$(1) \quad L = E_{mt}^{(-Ctmt)} / E_n^{(-Ctn)}$$

L – mtDNA level per cell

E_{mt} - PCR efficiency of mitochondrial target

E_n - PCR efficiency of nuclear target

Ct_{mt} - threshold cycle of mitochondrial target (point where the instrument first detects fluorescence above bacgkround noise)

Ct_n - threshold cycle of nuclear target

3.11 mRNA analysis

To quantify transcript levels of analysed genes, we firstly selected two reference genes from group of six candidate genes (*ATP6* (ATP synthase subunit 6), *ATP5O* (ATP synthase subunit O), *SDHA* (succinate dehydrogenase complex, subunit A), *HPRT1* (hypoxanthine phoshoribosyltransferase 1), *TBP* (TATA box binding protein 1), *PSMB6* (protesome beta 6 subunit)). It was performed by GeNorm application, a Microsoft Excel programme available at http://medgen.ugent.be/wjvdesomp/genorm/. Reference genes were calculated from the raw expression data. The expression stability (M) is determined so that the lowest M value is for the most stable gene and the gene with the highest instability has the highest M value. Then the specific normalization factors were calculated using the geometric mean to control for changes in relative gene expression and outlying values (Vandesompele, et al., 2002). The equation (2) was used to determine the normalized mRNA expression levels of all selected genes.

$$(2) \quad L_e = E_{goi}^{(-Ctgoi)} / NF$$

L_e – normalized mRNA expresion level

E_{goi} - PCR efficiency of gene of interest

NF - normalization factor (individual for each sample)

Ct_{goi} - threshold cycle of gene of interest

3.12 Evaluation of the real-time PCR sensitivity

Sensitivity was determined by the Ct values which were plotted as a function of the DNA amount or the total RNA amount which was transcribed to cDNA (in nanograms) and then the limits of quantification and detection were assessed.

The limits of quantification were in range 10 pg – 100 ng of genomic DNA for mtDNA quantification and 100 pg – 100 ng of genomic DNA for GAPDH quantification. 1 pg of genomic DNA for mtDNA quantification and 10 pg of genomic DNA for GAPDH quantification were determined as the limits of detection.

The limits of quantification were 50 ng – 1 ng (*POLG*, *TFAM*, *NRF1, COX4, MTCO2* and *PGC1A* quantification) or 200 – 2 ng (*NRF2, PPRC1* and *PGC1B* quantification) of total RNA. The limits of detection were 0.4 ng (*TFAM*, *POLG*, *NRF1, COX4, MTCO2* and *PGC1A* quantification) or 0.2 ng (*NRF2, PPRC1* and *PGC1B* quantification) of total RNA.

Inter-assay plate variation was < 0.5 ΔCt for every gene tested.

3.13 Statistical Analysis

Cts were calculated with Opticon 3 Software (Bio-Rad) or 7300 System Software (Applied Biosystems). The baseline value was the average fluorescence value over cycle range 3 – (n_{Ct} – 2; n = number). Pearson´s correlation, linear regression and Cook´s distance analyses were used to evaluate the relationships among mtDNA content, *TFAM*, *POLG*, *NRF1*, *NRF2, PGC1A, PGC1B, PPRC1, MTCO2, COX4, ATP6, ATP5O, ATP5G2* mRNA expression levels and gestational age. The test of correlation coefficient significance was also performed. All statistical analyses were provided by STATISTICA software, version 6.0 (StatSoft, Prague, CZ). Results were considered significant when the corresponding $P < 0.05$.

Table 6. Primer sequences and their characteristics.

Primer name	oligonucleotide sequence (5´- 3´) forward / reverse	Annealing temperature/ time	Amplicon lenght	GenBank accesion number
GAPDH	TTCAACAGCGACACCCACT/ CCAGCCACATACCAGGAAAT	60°C / 15s	95 bp	NG_007073
MT-RNR2	CCAAACCCACTCCACCTTAC/ TCATCTTTCCCTTGCGGTA	58°C / 15s	118 bp	NC_001807
POLG	CAGCCACAGCCAGCAAGT/ GAAACTCCTCCTCCTCACTGC	63°C / 20s	99 bp	NM_002693
TFAM	CCGAGGTGGTTTTCATCTGT/ GTTTTTGCATCTGGGTTCTGA	56 °C/ 20s	116 bp	AK312558
HPRT1	GACACTGGCAAAACAATGC/ AACACTTCGTGGGGTCCTTT	60°C / 20s	105 bp	NM_000194
TBP	TGCTCACCCACCAACAAT/ TCCAGAAACAAAAATAAGGAGAA	60°C / 20s	104 bp	NM_003194
PSMB6	TTTCCACAGCATTGAACTGA/ TACACCTGCCCTCCTTCTT	60°C / 20s	144 bp	AK312558
NRF1	AGAACTGCCGCCTCTCAC/ GGTTTTCCCCGACCTGTAG	63°C / 20s	117bp	NM_001040110
PGC1A	CAGAGAACAGAAACAGCAGCA/ TGGGGTCAGAGGAAGAGATAAA	58°C / 20s	116bp	NM_013261
MTCO2	TCATTTTCCTTATCTGCTTCC/ ACGGTTTCTATTTCCTGAGC	58°C / 20s	103bp	NC_012920.1
COX4	ATGTCAAGCACCTGTCTGC/ CCCTGTTCATCTCAGCAAA	57°C / 18s	135bp	NM_001861.2
ATP5O	TGAGAGTAGCACAAATCCTGA/ TGGTAGTGAGGGGAGAGAAC	57°C / 30s	132bp	NM_001697.2
MT-ATP6	CCACCCTAGCAATATCAACC/ GTGAAAACGTAGGCTTGGAT	60°C / 20s	115bp	NC_012920.1
ATP5G2	GCCTGCTCCAAGTTTGTCTC/ GTATCTCCGGTCGTTTCAGC	59°C / 20s	100bp	NM_001002031.2

All primers were design using Primer3 program (Rozen and Skaletsky, 2000) and synthesized by company Generi Biotech.

4 Results and discussion

4.1 Appropriate conditions for handling and storage of fetal tissue samples with a focus on effect on analysis of the mRNA expression

This pilot study was focus on evaluation of appropriate options for handling and storage of placenta tissue and fetal tissue samples. Human placenta represents easily obtainable source of human fetal tissue. Therefore it was used for evaluation basic rules for manipulation with fetal tissues. The most important requirement for successful real-time PCR is equal RNA quality in all of samples.

Apart from RNA quality, the choice of a proper set of reference genes for accurate normalization is another crucial factor with a profound impact on the reliability of the obtained gene expression levels (Pérez-Novo, et al., 2005). Therefore the reference genes for placental, fetal muscle and liver tissues were determined.

No study has been done with focus on Apgar score. This could be important factor because the lower is Apgar score, the higher is risk of probability that the fetus passes the hypoxemia. Then also the RNA could have inaprropriate quality for objective study. Therefore, in this pilot study, the Apgar score was observed in context of RNA purity, RNA quantity and RNA integrity (Table 7).

Table 7. RNA of human placenta tissue samples

Sample No	RNA quantity (ng/µ)	RNA purity	tissue weight (mg)	RIN
1	**4290**	**1.62**	**590**	**5.3**
2	**4472**	**1.92**	**440**	**4.8**
3	**4411**	**1.52**	**830**	**5.2**
4	**4464**	**1.98**	**680**	**5.4**
5	655	1.96	360	6.9
6	2105	1.98	100	4.1
7	4155	1.72	470	4.7
8	4286	1.63	550	4.9
9	3407	1.92	430	5.7
10	**4401**	**1.52**	**300**	**N/A**
11	4223	1.70	270	5.7
12	2400	1.97	290	6.3
13	453	1.88	360	7.2
14	1655	1.97	190	6.3
15	775	1.93	470	5.4
16	2958	1.95	210	4.9
17	1819	1.97	300	5.9
18	3518	1.88	470	6.4
19	3814	1.83	270	3.0
20	2204	1.99	160	3.8

The numbers in bold marks the low Apgar samples (3-7 points for the first interval). Samples No.1-9 are samples from Caesarean sections and No.10-20 are samples from spontaneous deliveries.

RIN – RNA integrity number

4.1.1 RNA purity (protein contamination) and quantity

The significant negative correlation was observed between RNA quantity and RNA purity of the placenta tissue samples ($r = -0.61$, $p < 0.01$; $y = 12845.19 - 5332.21x$) (Figure 7). The purity of RNA samples was 1.9 ± 0.1 (A260 / A280 ratio). There was also found the significant positive correlation between RNA quantity and sample weight ($r = 0.46$, $p < 0.05$; $y = 207.87 + 0.06x$). This result confirmed that isolation of RNA from placenta tissue is effortless.

In the time course study the connectivity was found also between RNA quantity and RNA purity for 0°C in the first ($r = -0.94$, $p < 0.05$; $y = 22677.73 - 10181.37x$) and the second ($r = -0.8384$, $p < 0.05$; $y = 28653.18 - 13366.12x$) intervals (Table 8, 9). It means that the higher is RNA amount the lower is RNA purity. According the result RNA quality especially purity is dependent on the temperature. This dependence was invalid for both temperatures 0 °C / 24 °C in the third interval ($p > 0.05$) (Table 10). It was probably influenced by number of samples. On the other hand the role of time period before freezing was also crucial as showed the analysis of RNA integrity. The low Apgar score did not affect RNA quantity. On the other hand RNA purity of low Apgar samples was the worst in the set of samples.

The average RNA purity for fetal tissues was 1.9 ± 0.1.

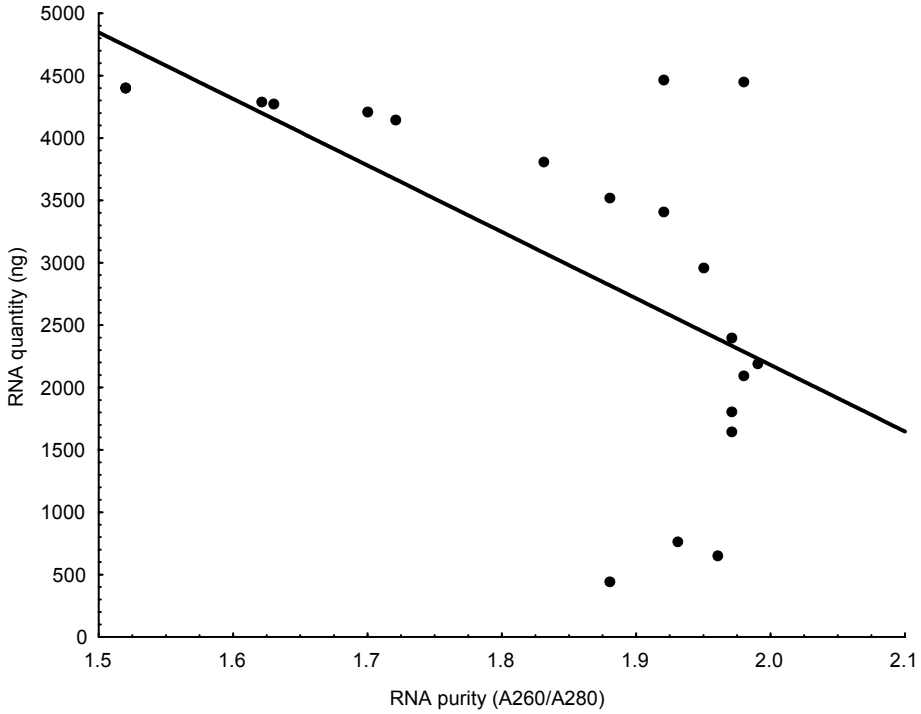

Figure 7. Correlation between RNA quantity and RNA purity of placenta tissue samples.

Table 8. The first interval (state after 1 hour)

Sample No	RNA quantity (ng/µ)	RNA purity	Tissue weight (mg)	RIN
6	3137	1.96	250	N/A
6	2491	2.00	270	5
12	843	1.92	250	5.3
12	3649	1.89	300	5.8
2	**2059**	**1.96**	**310**	**2.7**
2	**2960**	**1.92**	**450**	**4.9**
18	3276	1.91	430	4.9
18	3373	1.89	400	6.7
4	**3181**	**1.94**	**290**	**4.5**
4	**2360**	**1.98**	**310**	**4.4**

White fields marks 0°C and grey fields marks 24°C, the numbers in bold show the low Apgar samples.

Table 9. The second interval (state after 2 hours)

Sample No	RNA quantity (ng/µ)	RNA purity	Tissue weight (mg)	RIN
6	3791	1.86	620	2.3
6	3791	1.87	450	2.9
12	2832	1.98	290	4.8
12	3517	1.92	330	5.8
2	**2978**	**1.91**	**440**	**2.9**
2	**3755**	**1.84**	**390**	**3.3**
18	1721	1.97	240	3.5
18	1474	1.97	320	6
4	**3817**	**1.83**	**380**	**3.8**
4	**2619**	**1.97**	**250**	**5.4**
14	1020	1.96	100	3.6
14	2518	1.97	230	6.6

White fields marks 0°C and grey field marks 24°C, the numbers in bold show the low Apgar samples.

Table 10. The third interval (state after 10 hours)

Sample No	RNA quantity (ng/µ)	RNA purity	Tissue weight (mg)	RIN
6	1750	2.00	550	N/A
6	1228	2.00	360	2.7
12	443	1.93	230	2.3
12	1372	1.96	260	2.5
4	**1858**	**1.82**	**230**	**2.5**
4	**3770**	**1.84**	**360**	**2.6**

White fields marks 0°C and grey fields marks 24°C, the numbers in bold show low Apgar samples.

4.1.2 RNA purity (gDNA contamination)

All of placental samples were found without gDNA contamination, because only shorter *MECP2* gene fragment (378 bp) was amplified (Figure 8B). The longer fragment (1143 bp) was amplified from gDNA and therefore it was marker of gDNA contamination in RNA sample (Figure 8A). Significant differences were found in amplification of shorter *MECP2* gene fragment (Figure 8B). These differences correspond with temperature (0 °C or 24 °C) and also with time period before freezing of the samples. RNA from tissue sample which was placed in the lower temperature (0 °C) provided better PCR result. Moreover the shorter time interval (1 hour) showed still better PCR result.

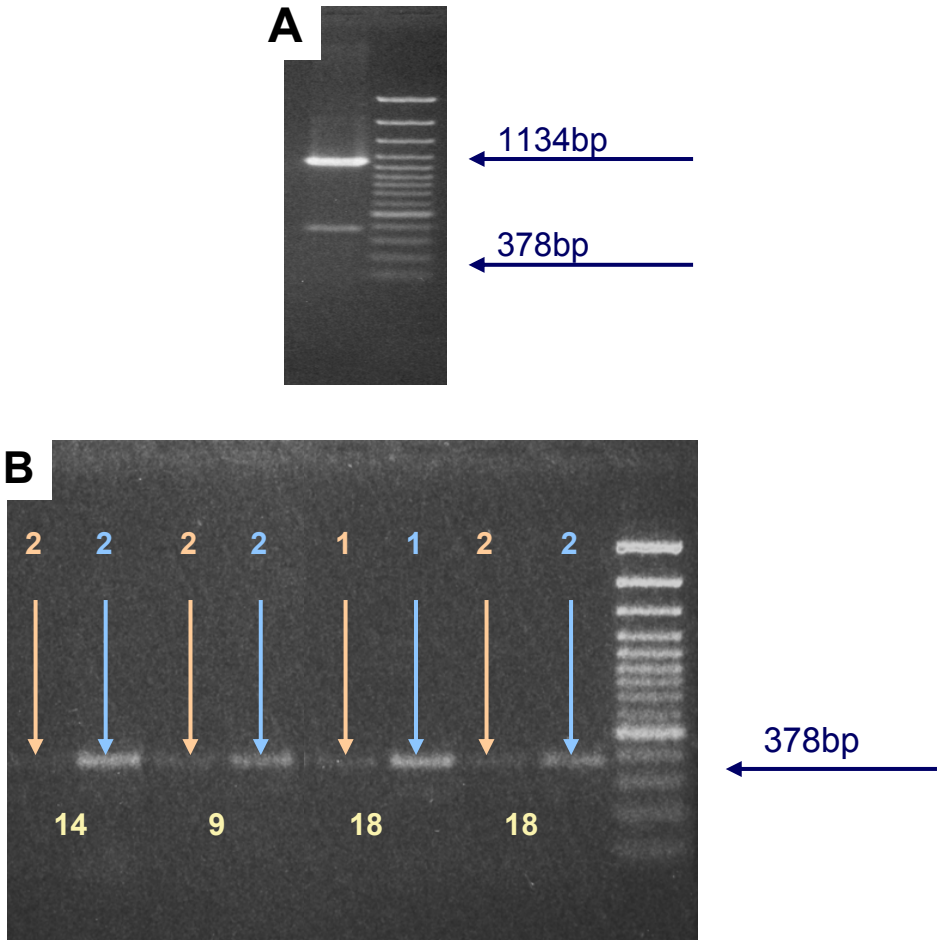

Figure 8. Analysis of gDNA contamination and changes between the intervals

A – sample No.14 with added gDNA (two fragments of MECP2 – 1134 bp from gDNA and 378 bp from transcribed RNA respectively cDNA), B – amplification of MECP2 fragment (378 bp) in the samples (No.9,14,18) of the first and the second intervals (blue arrows show 0°C and orange arrows show 24°C, the numbers above arrows mark intervals)

4.1.3 RNA integrity

The average RIN of 20 placental samples was 5.5 ± 1.0 and RINs of intervals were 5.4 ± 0.9 / 4.4 ± 1.1 (first interval 0 °C / 24 °C), 5.0 ± 1.5 / 3.5 ± 0.9 (second interval 0 °C / 24 °C), 2.6 ± 0.1 / 2.4 ± 0.1 (third interval 0 °C / 24 °C). It is evident that the longer is the time period before freezing the lower is RNA integrity. Moreover also the temperature has impact on RNA integrity (Figure 9). The RNA integrity of low Apgar samples was not significantly different from the integrity of normal Apgar samples.

The average RINs of 26 fetal muscle and 26 liver samples were 6.4 ± 1.6 and 6.6 ± 2.1.

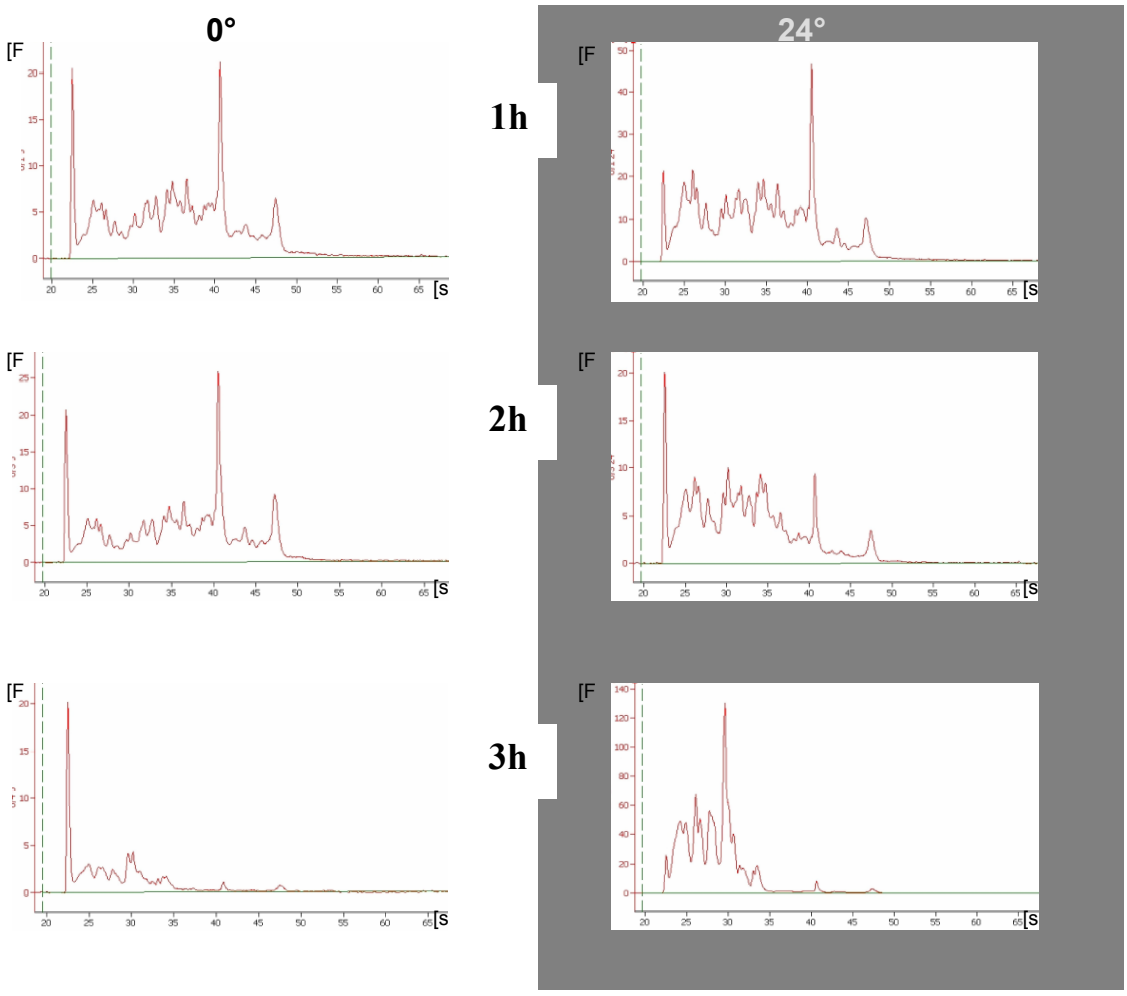

Figure 9. Differences in rRNA peaks among the intervals

The 18S rRNA and 28S rRNA peaks (40s and 48s) of sample No 12 in the three time intervals and two different temperatures (0 °C and 24 °C). When peaks disappear it means that the RNA integrity decreases. RINs were 5.8 / 5.3 (after 1 hour), 5.8 / 4.8 (after 2 hours), 2.5 / 2.3 (after 3 hours).

4.1.4 Evaluation of appropriate reference genes

In placenta tissue, *ATP50* and *SDHA* were the most stable expressed with M = 0.340 (Figure 10). Similar result for placenta tissue was shown in study Meller et al. (2005).

TBP and *PSMB6* were the most stable expressed genes in fetal liver tissue with an average expression stability (M) of M = 0.287. *HPRT1* and *PSMB6* were the most stable expressed genes in fetal muscle tissue with an average expression stability of M = 0.255.

4.1.5 Summary

According to the results, the important factors for handling, storage and acquirement of the best RNA quality of human placenta and fetal tissue samples are temperature and time period before freezing of samples. Therefore the proper conditions are storage in 0 °C for 1 hour maximally or immediately to freeze in – 80 °C and RIN at least 4.5 - 5.

Apgar score, which could be often depend on the process of childbirth (Caesarean section or spontaneous childbearing), is relevant only for placenta tissue samples. The lower Apgar score may negatively influence the probability of impurities in RNA from placenta tissue.

The findings were worth because the evaluation of proper rules are necessery for manipulation with fetal tissues. Therefore the results helped me to realize the next aims of this work.

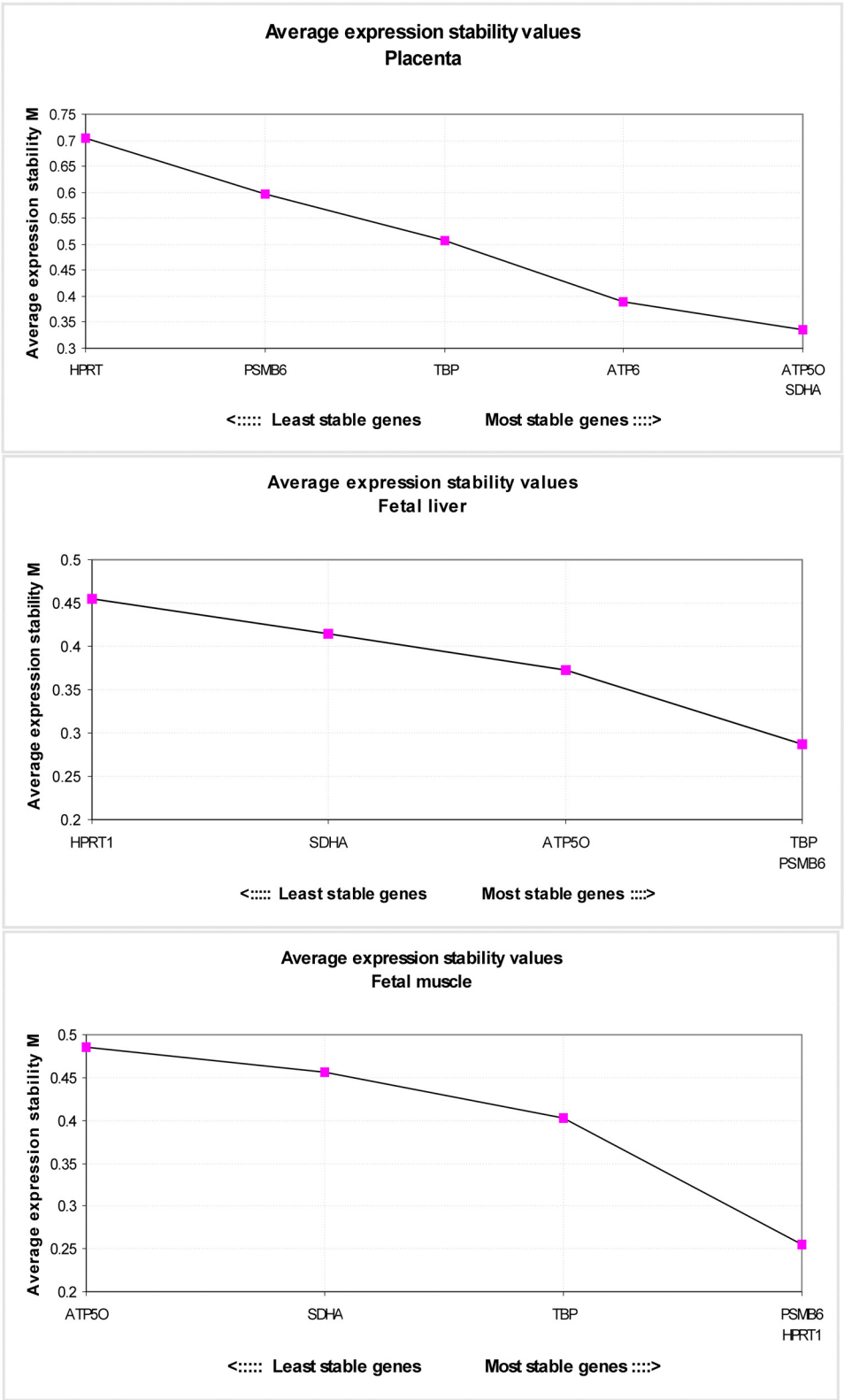

Figure 10. GeNorm analysis of the most stable genes in fetal tissues

The six genes (*ATP6, ATP5O*, *SDHA*, *HPRT1*, *TBP* , *PSMB6)* were tested in placenta tissue (n = 12) and five genes *(ATP6*, *SDHA*, *HPRT1*, *TBP* , *PSMB6)* in fetal muscle and liver tissues (n = 10)

4.2 The mtDNA content changes in human cord blood leukocytes (HCBL), fetal liver and muscle tissues during second trimester of gestation

This work demonstrates a progressive increase in the ratio of mitochondrial to nuclear DNA in both fetal liver and fetal muscle tissues during the second trimester of gestation.

On the contrary, in HCBL, the mtDNA content gradually decreases between 25 – 41 weeks of gestation. Isolated HCBL contribute very little to the overall metabolic turnover, but they may serve as easily available marker cells for the study of the mtDNA amount changes in cord blood during gestation (Pejznochova, et al., 2008).

4.2.1 The changes of mtDNA content in HCBL during gestation

The significant negative correlation was found between the mtDNA content in HCBL and gestational age in the set of 107 neonates born between 25 – 41 weeks of gestation. Using the raw data, correlation analysis gave a Pearson's correlation coefficient of $r = - 0.54$ ($p < 0.01$) (Figure 11C). The relative mtDNA level (mean ± SD) was 69.7 ± 9.9. The correlation analysis only for gestational age 37 – 41 weeks gave a Pearson´s correlation coefficient of $r = - 0.33$ ($p < 0.05$). Therefore the prematurity did not affect significantly our results. There was also the significant negative correlation between the mtDNA content and birth weight. The Pearson's correlation coefficient was $r = - 0.43$ ($p < 0.01$).

The mtDNA amount in HCBL is decreasing during the gestation contrary to the Heerdt and Augenlicht (1990) who described no significant differences in mtDNA amount between adult and umbilical blood leukocytes and bone marrow. However, in that study, mtDNA amount in leukocytes was analysed only from two umbilical blood samples and ten peripheral blood samples from adults. Moreover, the adult and fetal samples were compared using Southern blot method, which is less sensitive than real-time PCR.

Several reasons exist why the mtDNA amount could decrease in leukocytes during fetal development. Our study was performed between the sixth and the ninth fetal

month when the anatomic shift of hematopoiesis from the fetal liver to the bone marrow is proceeding (Baron, 2003; Brugnara and Platt, 2003). Simultaneously with this shift the number of hematopoietic progenitor cells (HPC) was proved to be decreasing. It was shown in premature neonate blood whose number of HPC is high and quickly fall in contrast to number of HPC in mature neonate blood (Brugnara and Platt, 2003; Haneline, et al., 1996; Wyrsch, et al., 1999). On the other hand, an upward trend in number of leukocytes was shown during the gestation (Forestier, et al., 1991). This trend could be caused by falling of the cell volume during the development as it was observed in erythrocytes (Forestier, et al., 1991; Tannirandorn, et al., 1999). In summary, the falling number of HPC, the growing number of leukocytes as marker of the cell parameter changes together with the overlap of the fetal liver and the bone marrow hematopoiesis could explain the decrease of mtDNA amount per cell in HCBL. Thus, the mtDNA level decrease in HCBL is the secondary effect of the physiological changes in the fetal hematopoiesis.

Platelets could influence the result of the mtDNA measurement because they contain mtDNA but no nDNA (Banas, et al., 2004). However, it was demonstrated that the count of platelets is constant during gestation (Forestier, et al., 1991). Therefore the observation of mtDNA content downtrend during gestation is not influenced by possible platelet contamination. The situation in reticulocytes is similar to platelets, the count of reticulocytes is not significantly changed during gestation (Brugnara and Platt, 2003).

In our study, the real-time PCR method was used because it is the most sensitive and accurate method for mtDNA quantification (Bhat and Epelboym, 2004; Chabi, et al., 2003). The real-time PCR was established to compare the relative abundance of mtDNA with nDNA. The nDNA amount is directly proportional to the number of cells and therefore the assay result could be expressed as mtDNA amount per cell. The GAPDH was selected as a reference for nDNA. It is an example of a single copy gene that acts as a marker of diploid genome content. Other non-single copy nuclear genes, especially genes for 28S rRNA or 18S rRNA, which have been often used in other studies (Bai, et al., 2004; Cha, et al., 2005; Song, et al., 2001), seem to be less appropriate. The repetitive nuclear sequences may vary in number and even in primary sequence from one individual to another (Gonzalez, et al., 1985) therefore they are not efficient both as an endogenous references and as markers of genome content.

Validation of the real-time PCR parameters, mainly efficiency, was provided by the calibration curve in each run for all experiments. The calibration curve was prepared from the HCBL genomic DNA, because only this genomic DNA has the identical characteristics (primarily efficiency) as genomic DNA of analysed samples.

4.2.2 Changes of mtDNA content in fetal liver and muscle during gestation

The significant positive correlations were found between the mtDNA amount and the gestational age in fetal muscle and liver tissues (muscle: $r = 0.56$, $p < 0.01$; liver: $r = 0.59$, $p < 0.01$, respectively, Figure 11A,B). The relative mtDNA level (mean ± SD) was higher in fetal muscle than in liver (liver: 130 ± 60; muscle 257 ± 134; $p < 0.001$) (Pejznochova, et al., 2010).

In the period between 13th and 28th week of gestation covered by our study the role of fetal liver as main hematopoietic site declines (Brugnara and Platt, 2003). Through this change the proliferation of the hepatocytes is rising simultaneously with mitochondrial proliferation and therefore the mtDNA content increases (Izquierdo, et al., 1995). Our results showed the significant increase of mtDNA content in fetal period as well as in the study of Heerdt and Augentlicht (1990). On the other hand, in the earlier fetal development (9th - 17th week of gestation) the liver mtDNA content is not significantly changed (Minai, et al., 2008). Further, the data of postnatal period showed notable increase of the mtDNA amount in comparison with the fetal values similarly in studies of Morten et al. (2007) and Minai et al. (2008). The prenatal mtDNA levels (mean ± SD) were significantly lower than the postnatal mtDNA levels (mean ± SD) in both tissues (postnatal liver: 1496 ± 716; postnatal muscle: 1358 ± 797; $p < 0.001$) (postnatal data was made by Ing. K.Vinsova together with me in our lab and data is not shown).

The terminally differentiated cells of human skeletal muscle are formed in three distinct waves. The first wave proceeds in embryonic period. The secondary muscle fibres begin to form in fetal period and the tertiary wave of myofibre formation continues past the time of birth (Miller, 2001). Between the first and the secondary waves, the mtDNA amount shows no change (Minai, et al., 2008). Nevertheless, the results of our work, mapping the mainstream of the secondary wave, revealed

significant increase in the mtDNA amount between 13th and 28th week of gestation. It might be induced as glycolytic energy production in proliferating myoblasts shifts to oxidative phosphorylation in differentiated myotubes (Franko, 2008; Leary, et al., 1998). Moreover musle differentiation is probably associated with the increase of mtDNA content during rat embryonic development (Franko, 2008; Miki, et al., 1988). Our analysis of the postnatal versus fetal muscle tissue showed the significant difference in mtDNA content. Similarly to study of Minai et al. (2008) the postnatal values were higher.

Although the significant increase in the mtDNA content was observed in both tissues during analysed period, a higher relative amount of mtDNA was found in the muscle compared to the liver. Furthermore, as mentioned above, the differences were observed between the postnatal and fetal mtDNA content in both analysed tissues. It means that after birth the increase of mtDNA content continues in both tissues. The non-homogeneous distribution of mtDNA between fetal tissues could be given by their different metabolic roles. The tissue-specific mtDNA content implies a tissue-specific expression of genes involved in maintenance and regulation of mtDNA.

4.2.3 Summary

Results of our studies demonstrated the significant changes of mtDNA amount in HCBL, fetal liver and muscle tissues during the second part of gestation. The highest amount of mtDNA was found in fetal muscle at the end of the analysed period. In the HCBL was found the minimal content of mtDNA in comparison with fetal liver and muscle. Therefore the results are in accordance with the hypothesis that in various human developing tissues the mtDNA amount could be different not only depending on their energy demand but also on the stage of development (Heerdt and Augenlicht, 1990).

The real-time PCR method was introduced for quantification of mtDNA. This approach to the relative DNA quantification is sensitive, accurate, and simple.

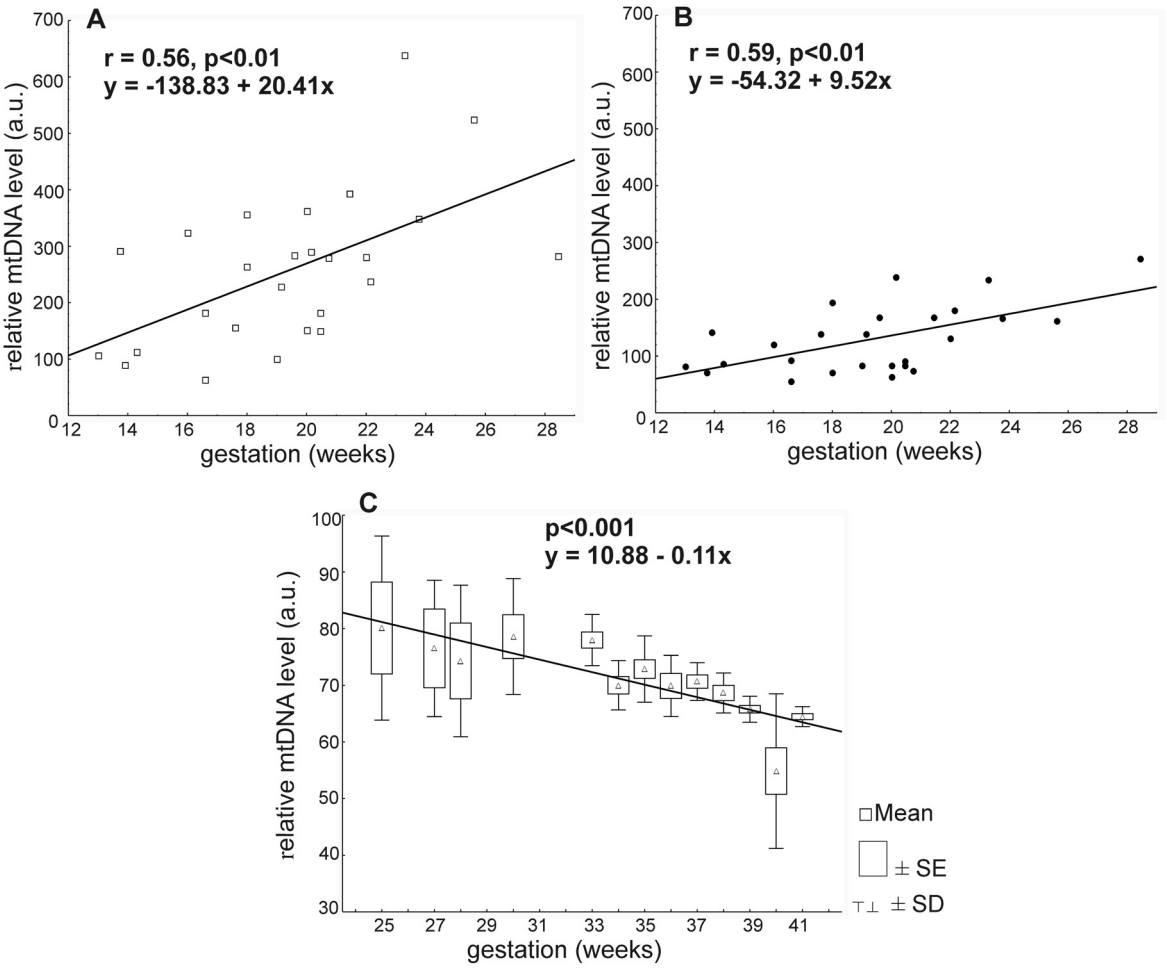

Figure 11. The correlation between the mtDNA level and the weeks of gestation in human fetal muscle (A), fetal liver (B) and cord blood leukocytes (C)

4.3 The trends in expression of the genes involved in mtDNA transcription, regulation and maintenance, in human fetal liver and muscle tissues between 13th and 28th week of gestation

The intriguing differences were found in expression of *POLG*, *TFAM*, *NRF2, MTCO2, COX4, ATP5G2, ATP6* genes and family *PGC1* coactivators between the fetal liver and muscle tissues (Table 12).

In both tissues, there is the trend to increase *TFAM* mRNA expression from 13th till 28th week of gestation (liver: $r = 0.47$, $p < 0.05$; muscle: $r = 0.63$, $p < 0.01$) (Figure 12). The results showed that mRNA level of *TFAM* follows the trend of mtDNA content in fetal muscle and liver tissues (liver: $r = 0.62$, $p < 0.01$; muscle: $r = 0.51$, $p < 0.01$). Similarly, in both tissues, a significantly positive correlation was observed between *TFAM* and *MTCO2* mRNA levels (liver: $r = 0.72$, $p < 0.01$; muscle: $r = 0.56$, $p < 0.01$) and between *TFAM* and *ATP6* mRNA levels (liver: $r = 0.49$, $p < 0.05$; muscle: $r = 0.67$, $p < 0.01$).

Interestingly, the significantly higher relative amount of the *TFAM* mRNA was observed in fetal liver (mean ± SD) (l4.10 ± 1.77) in comparison to fetal muscle (1.9 ± 0.50) ($p < 0.01$) although the higher mtDNA content was found in fetal muscle (Figure 11). This could signify more intensive mitochondrial proliferation on transcriptional level in liver than in muscle. *TFAM* mRNA level probably reflects the rate of mtDNA transcription (Ostronoff, et al., 1996). Therefore the analysis of the expression of mitochondrially encoded genes - *MTCO2* and *ATP6* was performed. Their mRNA levels are rapidly increasing during observed fetal period in both tissues (liver: $r = 0.65$, $p < 0.001$ and $r = 0.69$, $p < 0.001$; muscle: $r = 0.71$, $p < 0.001$ and $r = 0.73$, $p < 0.001$), but the overall *MTCO2* (Figure 12) and *ATP6* mRNA levels are higher in liver tissue. It supports our hypothesis about stimulation of mitochondrial proliferation on transcriptional level in fetal liver. However TFAM, apart from its role in the mtDNA transcription initiation and replication of the mitochondrial genome (Shadel and Clayton, 1993) is also important in mtDNA maintenance as a main component of the nucleoid (Kang, et al., 2007). Therefore *TFAM* mRNA level might be related also to the mtDNA content (Ekstrand, et al., 2004; Chow, et al., 2007). Based on own results in the fetal liver, the increasing level of *TFAM* transcript

confirms hypothesis that it precedes accelerated growth of the mtDNA content (May-Panloup, et al., 2005) and supports the rise of the mtDNA transcription rate.

POLG encodes a catalytic subunit of polymerase gamma (Graziewicz, et al., 2006). The observations of our work bear the fact that *POLG* expression is tissue specific (Schultz, et al., 1998). *POLG* level is significantly increasing in fetal liver whereas it is not changed in muscle tissue during the analysed period (liver: $r = 0.63$, $p < 0.001$; muscle: $r = -0.38$, $p > 0.05$). In the liver, the increasing *POLG*, similarly to *TFAM*, could reflect the rate of mitochondrial proliferation (Ostronoff, et al., 1996). Moreover in mouse embryos, the up-regulation of liver mtDNA levels leads to increased levels of *POLG* transcripts (Hance, et al., 2005b). In rabbit skeletal muscle, the *POLG* transcript level was not shown to be related with the mtDNA content and its changes does not influence the abundance of the catalytic subunit (Schultz, et al., 1998).

The *TFAM* expression is regulated by NRF1 and NRF2, meanwhile NRF factors (NRFs) are affected by PGC1 family of regulated coactivators (PGC1A, PGC1B and PPRC1) (Cannino, et al., 2007; Choi, et al., 2006; Kelly and Scarpulla, 2004; Scarpulla, 2008b). PGC1A/B coactivate NRFs that in turn stimulate the expression of nuclear genes involved in mitochondrial respiration and biogenesis (Wu, et al., 1999). PPRC1 has been presented as NRF1 transcriptional coactivator (Andersson and Scarpulla, 2001). NRFs, DNA-binding transcription factors, have a broader role in the integration of diverse cellular functions (Huo and Scarpulla, 2001) including mtDNA maintenance (May-Panloup, et al., 2005) and transcription of nuclear encoded mitochondrial genes (Patti, et al., 2003; Scarpulla, 2008a). In both fetal tissues, the mRNA level of *PPRC1* keeps constant (liver: $r = 0.09$, $p > 0.05$; muscle: $r = 0.18$, $p > 0.05$) whereas the *PGC1B* level increases (liver: $r = 0.50$, $p < 0.05$ and muscle: $r = 0.58$, $p < 0.01$). Conversely the *PGC1A* transcript is changed entirely in liver (liver: $r = 0.47$, $p < 0.05$; muscle: $r = 0.14$, $p > 0.05$). Similarly the *NRF1* transcript was without variations in both tissues (liver: $r = 0.32$, $p > 0.05$; muscle: $r = 0.19$, $p > 0.05$), but the *NRF2* mRNA level rises significantly only in liver (liver: $r = 0.58$, $p < 0.01$; muscle: $r = -0.23$, $p > 0.05$). The transcript of nuclear encoded mitochondrial gene – *COX4*, showed also the tissue-specific increase (liver: $r = 0.67$, $p < 0.001$; muscle: $r = 0.20$, $p > 0.05$). Interestingly, the interdependence between *NRF2* and *COX4* transcript levels or between *NRF2* and *PGC1A* transcript levels was significantly positive only in fetal liver (liver: $r = 0.85$, $p< 0.01$; muscle: $r = 0.03$, $p > 0.05$ or liver: $r = 0.48$, $p < 0.01$; muscle: $r = 0.29$, $p < 0.05$). In accordance with

Puigserver (2005), it could bear evidence of tissue-specific expression or regulation. According to these results difference in the *PGC1A* transcript level between liver and muscle could explain tissue variation in *NRF2*, *TFAM* and *COX4* expression. The increasing expression of *PGC1A* positively affected NRFs binding to regulatory regions of the TFAM promoter (Wu, et al., 1999). Further NRF2 stimulates the trancription of mitochondrial nuclear encoded *COX4* gene (Ongwijitwat, et al., 2006; Ongwijitwat and Wong-Riley, 2005; Virbasius, et al., 1993) and TFAM increases transcription of mitochondrially encoded *MTCO2* and *ATP6* genes. It means that the mitochondrial proliferation in liver is regulated mainly on transcriptional level. On the contrary, in muscle it could be proceed rather on post-transcriptional or translational level as was shown in *POLG* expression (Schultz, et al., 1998).

The other evidences of tissue-specific expression respectively regulation of expression are differences in *ATP5G2* transcript levels between fetal tissues (liver: $r = 0.59$, $p < 0.01$; muscle: $r = 0.23$, $p > 0.05$). Moreover the next differences were found in interdependencies of *ATP5G2* and mtDNA or *ATP5G2* and *ATP6* transcript levels between fetal liver and muscle tissues. In details, there was found significantly positive correlation between transcript level of nuclearly encoded gene *ATP5G2* and mtDNA content in fetal liver tissue, but not in fetal muscle tissue (liver: $r = 0.53$, $p < 0.01$; muscle: $r = 0.23$, $p > 0.05$). Further the significantly positive correlation was found between transcript levels of nuclearly encoded gene *ATP5G2* and mitochondrially encoded gene *ATP6* in fetal liver, but not in fetal muscle (liver: $r = 0.79$, $p< 0.01$; muscle: $r = -0.10$, $p > 0.05$). These correlations show that regulation of cross-talk between mitochondria and nucleus on mRNA level proceeds more actively in fetal liver during prenatal development. It is in accordance with the suggestion that the mitochondrial proliferation in liver is regulated mainly on transcriptional level.

ATP5O, ATP5G2 and ATP6 (subunits OSCP, c and a) are involved in formation of an energy transduction part of ATP synthase (complex V) called F_0 (Figure 2). *ATP5O* and *ATP5G2* genes belong to low transcript gene group (LTG) (Sangawa, et al., 1997). This fact was confirmed in this work – their transcript levels were from ten fold to thousand fold lower in comparison with *ATP6* transcript level in analysed fetal tissues. The transcriptional regulatory system of the LTG may play a key role in the biogenesis of mammalian complex V respectively was termed as bottleneck in the complex V biogenesis (Sangawa, et al., 1997). Thus the mitochondrial biogenesis

could be also affected by the regulatory system of the LTG. Moreover according Houstek et al. (1995) the transcript level of *ATP5G2* gene (subunit c) correlates with the protein content of complex V. Together with our results, the amount of complex V probably increases in fetal liver during the second trimester.

4.3.1. Summary

The significant positive correlations were found between mRNA expression levels of *TFAM* and the gestational age in both fetal liver ($r = 0.46$, $p < 0.05$) and muscle tissues ($r = 0.62$, $p < 0.01$ or $r = 0.71$, $p < 0.01$) (Figure 12). The analogous correlations were found for mRNA level of *PGC1B* in both fetal tissues (liver: $r = 0.50$, $p < 0.05$ and muscle: $r = 0.58$, $p < 0.01$). Further mRNA expression level of *POLG*, *NRF2*, and *PGC1A* positively correlated with the gestation age only in fetal liver tissue ($r = 0.63$, $p < 0.001$; $r = 0.58$, $p < 0.01$; $r = 0.47$, $p < 0.05$). No correlations were between mRNA expression levels of *NRF1* or *PPRC1* and the gestational age in both fetal liver and muscle tissues.

The notable changes were observed in expression of selected COX and ATP synthase subunits on mRNA level (*COX4, ATP5O, ATP5G2* and *ATP6, MTCO2*) (Table 12). The mRNA levels of *COX4* (Figure 12) and *ATP5G2* were increasing only in liver ($r = 0.67$, $p < 0.001$ and $r = 0.59$, $p < 0.01$), meanwhile the transcript levels of *MTCO2* (Figure 10) and *ATP6* were raising in both tissues during observed fetal period (liver: $r = 0.65$, $p < 0.001$ and $r = 0.69$, $p < 0.001$; muscle: $r = 0.71$, $p < 0.001$ and $r = 0.73$, $p < 0.001$). The *ATP5O* transcript levels were without significant changes in both tissues (The analysis of ATP synthase subunits in fetal liver tissue was made by student Bc. A. Mrhalkova working with me in our lab). The analysis of gene expression was realized in the same set of samples as the analysis of mtDNA content.

Table 12. The correlations of mRNA expression levels or mtDNA content and gestation age.

	Muscle				Liver			
	r	p	n	equation	r	p	n	equation
PGC1A	0.14	>0.05	26	y = 0.34 + 0.02x	**0.47**	**<0.05**	**26**	**y = -0.06 + 0.03x**
PGC1B	**0.58**	**<0.01**	**26**	**y = - 18.18 + 1.33x**	**0.50**	**<0.05**	**26**	**y = - 6.73 + 0.59x**
PPRC1	0.18	>0.05	25	y = 2.03 + 0.14x	0.09	>0.05	26	y = 5.33 + 0.08x
NRF2	-0.23	>0.05	25	y = 16.26 – 0.35x	**0.58**	**<0.01**	**25**	**y = - 82.63 + 5.10x**
NRF1	0.19	>0.05	26	y = 0.30 + 0.01x	0.32	>0.05	25	y = 0.11 + 0.01x
TFAM	**0.63**	**<0.001**	**25**	**y = 0.08 + 0.09x**	**0.47**	**<0.05**	**26**	**y = - 0.23 + 0.22x**
MTCO2	**0.71**	**<0.001**	**23**	**y = -822.56 + 60.32x**	**0.65**	**<0.001**	**26**	**y = - 13352.60 + 808.14x**
COX4	0.20	>0.05	26	y = 31.32 + 0.54x	**0.67**	**<0.001**	**25**	**y = - 30.13 + 3.55x**
POLG	-0.38	>0.05	25	y = 0.70 – 0.01x	**0.63**	**<0.001**	**25**	**y = - 0.63 + 0.08x**
ATP5O	0.20	>0.05	26	y = 10.94 + 0.39x	-0.02	>0.05	26	y = 2.05 - 0.01x
ATP5G2	0.23	>0.05	26	y = 10.95 + 0.35x	**0.59**	**<0.01**	**25**	**y = -4.67 + 0.50x**
ATP6	**0.73**	**<0.001**	**24**	**y = -711.93 + 53.70x**	**0.69**	**<0.001**	**24**	**y = -14814.99 + 885.10x**

n – number of samples
The grey fields show significantly positive correlations

Table 13. The interdependences among RNA expression levels of studied genes and/or mtDNA level.

	Muscle			Liver		
	r	**p**	**n**	**r**	**p**	**n**
TFAM/mtDNA	**0.51**	**<0.01**	**25**	**0.62**	**<0.01**	**25**
TFAM/MTCO2	**0.72**	**<0.01**	**23**	**0.56**	**<0.01**	**25**
TFAM/ATP6	**0.67**	**<0.01**	**25**	**0.49**	**<0.05**	**22**
NRF2/COX4	0.03	>0.05	23	**0.85**	**<0.01**	**23**
NRF2/PGC1A	0.29	>0.05	23	**0.48**	**<0.05**	**23**
ATP5G2/ATP6	-0.10	>0.05	26	**0.79**	**<0.01**	**26**
ATP5G2/mtDNA	0.23	>0.05	26	**0.53**	**<0.01**	**26**

n – number of samples
The grey fields show significantly positive correlations

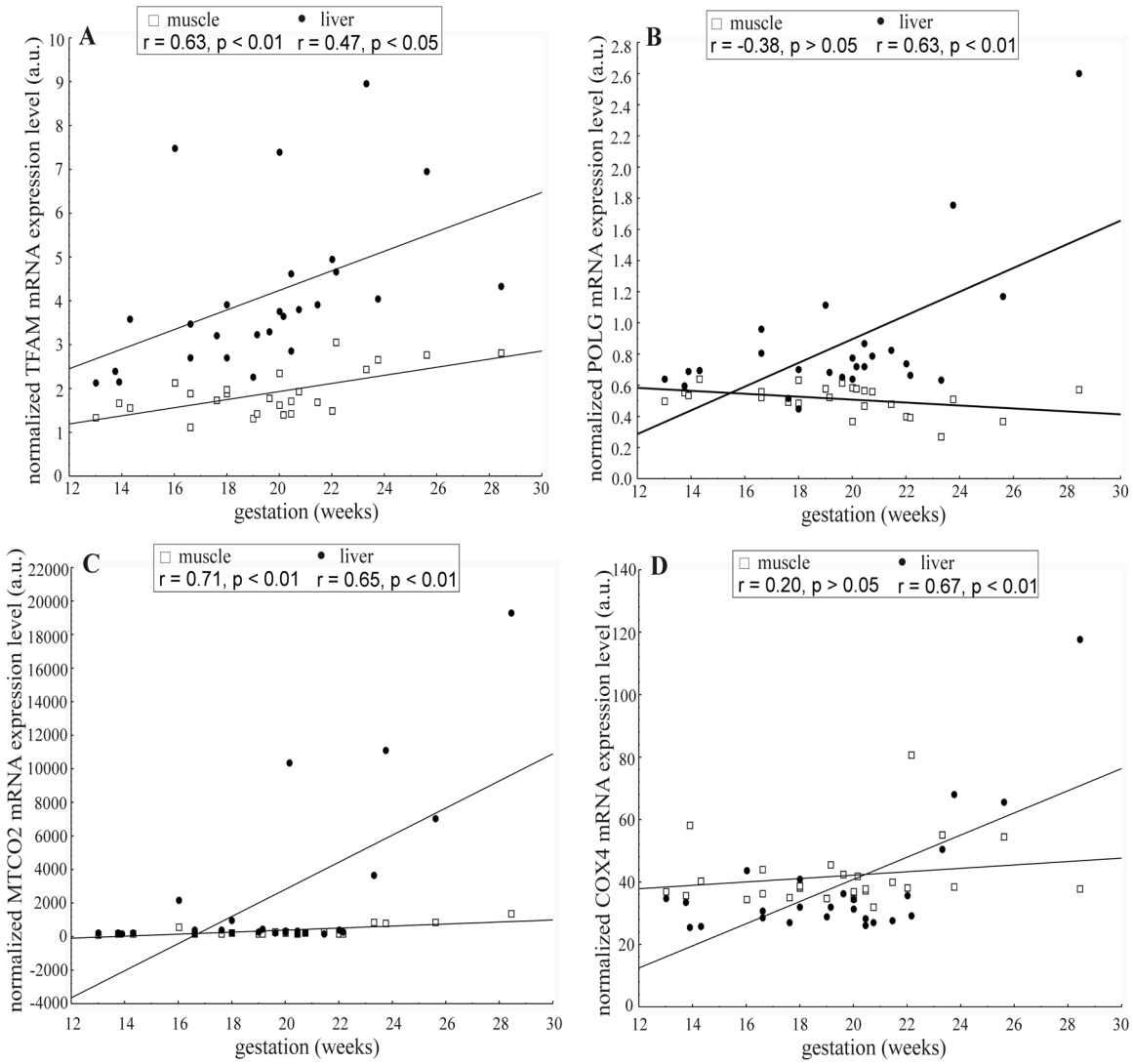

Figure 12. The correlations between *TFAM*, *POLG*, *MTCO2*, *COX4* normalized mRNA expression levels and the weeks of gestation. In fetal muscle tissue, the *TFAM* mRNA level was increasing but the *POLG* mRNA level was decreasing during period. The only mitochondrially encoded COX subunit – *MTCO2* was significantly raising, meanwhile *COX4* transcript level showed no changes through the period. In fetal liver tissue, the *TFAM* , *POLG*, *MTCO2* and *COX4* mRNA levels were raising during the period.

5 CONCLUSIONS

The specific conclusions of this study are:

a) The appropriate conditions for handling and storage of fetal tissue samples were evaluated. The basic essential parameters, which are important to observe in analysis of RNA from fetal tissues, were specified. They include first of all low temperature (ideally 0°C), short time period before freezing of samples (maximally 1h) and optimal RNA integrity (minimal RIN = 4.5).

b) The real-time PCR method was established for quantification of mtDNA content in fetal tissues and HCBL. The method is also available for other aplications e.g. as screening tool for Alpers syndrom which significantly change mtDNA content. Further the changes of mtDNA content were characterized in three different tissues during human fetal development.

c) The study characterized the trends of gene expression in the pathway leading from *PGC1* family of regulated coactivators through *NRF1, NRF2, TFAM*, *POLG* to *COX4, MTCO2* and *ATP5O*, *ATP5G2*, *ATP6* genes in fetal liver and muscle tissues between 13^{th} and 28^{th} week of gestation. Moreover the gene expression results were compared with the results from quantification of mtDNA content. According to the results, the mitochondrial proliferation is increasing during the second trimester. The first hallmarks of adjustment for postnatal adaptation are already evident mainly on transcriptional level in the fetal period and it represents a step to the more flexible posttranscriptional regulation early after birth (Cuezva, et al., 1997). The fetal developing tissues, especially liver and muscle, differ in the control of mitochondrial biogenesis depending on their energy demand and the phase of development as well.

6 ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATP5G2	ATP synthase subunit 5
ATP5O	ATP synthase subunit 5
ATP6	ATP synthase subunit 6
Bp	base pair
DNA	deoxyribonucleic acid
cDNA	complementary DNA
COX	cytochrome c oxidase
COX4	cytochrome c oxidase subunit 4
CREB	cAMP response element binding
CSB	conserved sequence block
Cytb	cytochrome b
EDTA	ethylene diamine tetraacetic acid
$FADH_2$	flavinadenine dinucleotide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
HCBL	human cord blood leukocytes
Hg	hydrargyrum (mercury)
HMG	high mobility group
HPC	hematopoietic progenitor cells
HPRT1	hypoxanthine phoshoribosyltransferase 1
HPT	hypothalamic-pituitary-thyroid system
HSP	H-strand promoter
LSP	L-strand promoter
MECP2	methyl CpG binding protein 2
MEF2	myocyte enhancer factor-2
mRNA	messenger RNA
MT	metallothionein
MTCO2	cytochrome c oxidase subunit 2
mtDNA	mitochondrial DNA
MTERF	mitochondrial termination factor
MT-RNR2	mitochondrially encoded 16S RNA
NADH	nicotinamid adenine dinucleotide

nDNA	nuclear DNA
NRF	nuclear respiratory factor
O_H	H-strand origin
O_L	L-strand origin
OXBOX/REBOX	sequences present in promoter regions of nuclear genes encoding mitochondrial proteins
OXPHOS	oxidative phosphorylation system
PGC1	peroxisome proliferator-activated receptor gamma coactivator
POLG	polymerase gamma catalytic subunit
POLG2	polymerase gamma accesory subunit
POLRMT	mitochondrial RNA polymerase
PPRC	PGC1-related coactivator
PSMB6	protesome beta 6 subunit
RIN	RNA integrity number
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
SDHA	succinate dehydrogenase subunit A
SDS	sodium dodecyl sulphate
Sp1	specificity protein 1
T3	triiodothyronine
T4	thyroxine
TAS	termination associated sequence
TBP	TATA box binding protein 1
TCA	tricarboxylic acid cycle
TE	tris(hydroxymethyl)aminomethane EDTA
TFAM	transcription factor A
TFB	specificity factor
TH	thyroid hormone
TRE	thyroid hormone response element
TRH	thyrotropin releasing hormone
TSH	thyroid-stimulating hormone or thyrotropin
UTR	untranslated region
YY1	yin yang protein 1

Abbreviation in italic denotes gene.

7 REFERENCES

1. Albring, M., Griffith, J. and Attardi, G. (1977). Association of a Protein Structure of Probable Membrane Derivation with Hela Cell Mitochondrial DNA near Its Origin of Replication. PNAS 74**,** 1348–1352.
2. Alcolea, M. P., Colom, B., Llado, I., Garcia-Palmer, F. J. and Gianotti, M. (2007). Mitochondrial Differentiation and Oxidative Phosphorylation System Capacity in Rat Embryo During Placentation Period. Reproduction 134**,** 147-154.
3. Alcolea, M. P., Colom, B., Lladó, I., Gianotti, M. and García-Palmer, F. J. (2006). Mitochondrial Transcription Factor A (TFAM) Is Increased in Rat Embryo During Placentation and Associated with Mitochondrial Differentiation. Cell. Phys. Biochem. 17**,** 79-88.
4. Allen, J. F. (1993). Control of Gene Expression by Redox Potential and the Requirement for Chloroplast and Mitochondrial Genomes. J. Theor. Biol. 165**,** 609-631.
5. Andersson, U. and Scarpulla, R. C. (2001). Pgc-1-Related Coactivator, a Novel, Serum-Inducible Coactivator of Nuclear Respiratory Factor 1-Dependent Transcription in Mammalian Cells. Mol. Cell. Biol. 21**,** 3738-3749.
6. Arnon, S., Aviram, R., Dolfin, T., Regev, R., Litmanovits, I., Tepper, R. and Elpeleg, O. N. (2002). Mitochondrial DNA Depletion Presenting Prenatally with Skin Edema and Multisystem Disease Immediately after Birth. Prenat. Diagn. 22**,** 34-37.
7. Asin-Cayuela, J. and Gustafsson, C. M. (2007). Mitochondrial Transcription and Its Regulation in Mammalian Cells. Trends Biochem. Sci. 32**,** 111-117.
8. Bai, R.-K., Perng, C.-L., Hsu, C.-H. and Wong, L.-J. C. (2004). Quantitative PCR Analysis of Mitochondrial DNA Content in Patients with Mitochondrial Disease. Ann. New York Acad. Sci. 1011**,** 304-309.
9. Banas, B., Kost, B. and Goebel, F. (2004). Platelets, a Typical Source of Error in Real-Time PCR Quantification of Mitochondrial DNA Content in Human Peripheral Blood. Cells. Eur. J. Med. Res. 9**,** 371-377.
10. Baron, M. H. (2003). Embryonic Origins of Mammalian Hematopoiesis. Exp. Hematol. 31**,** 1160-1169.
11. Barrientos, A., Casademont, J., Cardellach, F., Ardite, E., Estivill, X., Urbano-Marquez, A., Fernandez-Checa, J. and Nunes, V. (1997a). Qualitative and Quantitative Changes in Skeletal Muscle MtDNA and Expression of Mitochondrial-Encoded Genes in the Human Aging Process. Biochem. Mol. Med. 62, 165-171.
12. Barrientos, A., Casademont, J., Cardellach, F., Estivill, X., Urbano-Marquez, A. and Nunes, V. (1997b). Reduced Steady-State Levels of Mitochondrial RNA and Increased Mitochondrial DNA Amount in Human Brain with Aging. Mol. Brain. Res. 52**,** 284-289.
13. Bhat, H. and Epelboym, I. (2004). Quantitative Analysis of Total Mitochondrial DNA: Competitive Polymerase Chain Reaction Versus Real-Time Polymerase Reaction. J. Biochem. Mol. Tox. 18**,** 180-186.
14. Breall, J., Rudolph, A. and Heymann, M. (1984). Role of Thyroid Hormone in Postnatal Circulatory and Metabolic Adjustments. J. Clin. Invest. 73**,** 1418-1424.
15. Brown, T. and Clayton, D. (2006). Genesis and Wanderings: Origins and Migrations in Asymmetrically Replicating Mitochondrial DNA. Cell Cycle 5**,** 917-921.
16. Brown, T. A., Cecconi, C., Tkachuk, A. N., Bustamante, C. and Clayton, D. A. (2005). Replication of Mitochondrial DNA Occurs by Strand Displacement with Alternative Light-Strand Origins, Not Via a Strand-Coupled Mechanism. Gen. Dev. 19**,** 2466-2476.
17. Brugnara, C. and Platt, O. S. (2003). The Neonatal Erythrocyte and Its Disorders in Nathan, D. G., Orkin, S. K., Look, A. T. and Ginsburg, D. (Eds), Hematology of Infancy and Childhood, Saunders, pp. 19-26.
18. Burrow, G. N., Fisher, D. A. and Larsen, P. R. (1994). Maternal and Fetal Thyroid Function. N. Engl. J. Med. 331**,** 1072-1078.

19. Cannino, G., Liegro, C. M. D. and Rinaldi, A. M. (2007). Nuclear–Mitochondrial Interaction. Mitochondrion 7**,** 359-336.
20. Clayton, D. (2003). Mitochondrial DNA Replication: What We Know. IUBMB Life 55**,** 213-217.
21. Craig, E. A., Voisine, C. and Schilke, B. (1999). Mitochondrial Iron Metabolism in the Yeast *Saccharomyces Cerevisiae*. Biochem. J. 380**,** 1167–1173.
22. Crompton, M. (1999). The Mitochondrial Permeability Transition Pore and Its Role in Cell. Death. Biochem. J. 341**,** 233-249.
23. Cuezva, J., Ostronoff, L., Ricart, J., Heredia, M. L. d., Liegro, C. D. and Izquierdo, J. (1997). Mitochondrial Biogenesis in the Liver During Development and Oncogenesis. J. Bioenerg. Biomembr. 29**,** 365-377.
24. Cui, L., Jeong, H., Borovecki, F., Parkhurst, C. N., Tanese, N. and Krainc, D. (2006). Transcriptional Repression of PGC-1alpha By Mutant Huntingtin Leads to Mitochondrial Dysfunction and Neurodegeneration. Cell 127**,** 59-69.
25. Davis, P., Tillmann, H., Davis, F. and Wehling, M. (2002). Comparison of the Mechanisms of Nongenomic Actions of Thyroid Hormone and Steroid Hormones. J. Endocrinol. Invest. 25**,** 377-388.
26. de Zegher, F., Vanhole, C., Van den Berghe, G., Devlieger, H., Eggermont, E. and Veldhuis, J. (1994). Properties of Thyroid-Stimulating Hormone and Cortisol Secretion by the Human Newborn on the Day of Birth. J. Clin. Endocrinol. Metab. 79**,** 576-581.
27. Ekstrand, M. I., Falkenberg, M., Rantanen, A., Park, C. B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C. M. and Larsson, N.-G. (2004). Mitochondrial Transcription Factor a Regulates MtDNA Copy Number in Mammals. Hum. Mol. Genet. 13**,** 935-944.
28. Enders, A. C. and Schlafke, S. (1981). Differentiation of the Blastocyst of the Rhesus Monkey. Am. J. Anat. 162**,** 1-21.
29. Falkenberg, M., Larsson, N.-G. and Gustafsson, C. M. (2007). DNA Replication and Transcription in Mammalian Mitochondria. Ann. Rev. Biochem. 76**,** 679-699.
30. Fernandez-Silva, P., Enriquez, J. and Montoya, J. (2003). Replication and Transcription of Mammalian Mitochondrial DNA. Exp. Phys. 88**,** 41-56.
31. Fisher, D., Dussault, J., Sack, J. and Chopra, I. (1976). Ontogenesis of Hypothalamic-Pituitary-Thyroid Function and Metabolism in Man, Sheep, and Rat. Recent. Prog. Horm. Res. 33**,** 59-116.
32. Fisher, D. A. (2008). Thyroid System Immaturities in Very Low Birth Weight Premature Infants. Seminars in Perinatology Transient Hypothyroxinemia of Prematurity 32**,** 387-397.
33. Fisher, R. P., Lisowsky, T., Parisi, M. A. and Clayton, D. A. (1992). DNA Wrapping and Bending by a Mitochondrial High Mobility Group-Like Transcriptional Activator Protein. J. Biol. Chem. 267**,** 3358-3367.
34. Forestier, F., Daffos, F., Catherine, N., Renard, M. and Andreux, J. (1991). Developmental Hematopoiesis in Normal Human Fetal Blood. Blood 77**,** 2360-2363.
35. Franko, A., Mayer, S., Thiel, G., Mercy, L., Arnould, T., Hornig-Do, H.-T., Wiesner, R. J. and Goffart, S (2008). CREB-1alpha Is Recruited to and Mediates Upregulation of the Cytochrome C Promoter During Enhanced Mitochondrial Biogenesis Accompanying Skeletal Muscle Differentiation. Mol. Cel. Biol. 28**,** 2446-2459.
36. Gadaleta, M., Rainaldi, G., Lezza, A., Milella, F., Fracasso, F. and Cantatore, P. (1992). Mitochondrial DNA Copy Number and Mitochondrial DNA Deletion in Adult and Senescent Rats. Mut. Res. 275**,** 181-193.
37. Garcia, J., Ananthakrishnan, R. and Goldenthal, M. J. (2000). Heart Mitochondrial DNA and Enzyme Changes During Early Human Development. Mol. Cell. Biochem. 210**,** 47-52.
38. Garesse, R. and Vallejo, C. G. (2001). Animal Mitochondrial Biogenesis and Function: A Regulatory Cross-Talk between Two Genomes. Gene 263**,** 1-16.
39. Garnier, A., Fortin, D., Deloménie, C., Momken, I., Veksler, V. and Ventura-Clapier, R. (2003). Depressed Mitochondrial Transcription Factors and Oxidative Capacity in Rat Failing Cardiac and Skeletal Muscles. J. Phys. 551**,** 491-501.

40. Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van der Bliek, A. M. and Spelbrink, J. N. (2003). Composition and Dynamics of Human Mitochondrial Nucleoids. Mol. Biol. Cell 14**,** 1583-1596.
41. Gibson, B. W. (2005). The Human Mitochondrial Proteome: Oxidative Stress, Protein Modifications and Oxidative Phosphorylation. Int. J. Biochem. Cell Biol. 37**,** 927-934.
42. Gleyzer, N., Vercauteren, K. and Scarpulla, R. C. (2005). Control of Mitochondrial Transcription Specificity Factors (TFB1M and TFB2M) by Nuclear Respiratory Factors (NRF-1 and NRF-2) and PGC-1 Family Coactivators. Mol. Cell. Biol. 25**,** 1354-1366.
43. Gluckman, P., Sizonenko, S. and Bassett, N. (1999). The Transition from Fetus to Neonate--an Endocrine Perspective. Acta Paediatr. Suppl. 88**,** 7-11.
44. Gonzalez, I. L., Gorski, J. L., Campen, T. J., Dorney, D. J., Erickson, J. M., Sylvester, J. E. and Schmickel, R. D. (1985). Variation among Human 28s Ribosomal RNA Genes. PNAS 82**,** 7666–7670.
45. Gray, M. W. (1999). Evolution of Organellar Genomes. Curr. Opin. Gen. Dev. 9**,** 678-687.
46. Graziewicz, M. A., Longley, M. J. and Copeland, W. C. (2006). DNA Polymerase Gamma in Mitochondrial DNA Replication and Repair. Chem. Rev. 106**,** 383-405.
47. Guarente, L. and Mason, T. (1983). Heme Regulates Transcription of the Cyc1 Gene of *S. Cerevisiae* Via an Upstream Activation Site. Cell 32**,** 1279-1286.
48. Hance, N., Ekstrand, M. I. and Trifunovic, A. (2005a). Mitochondrial DNA Polymerase Gamma Is Essential for Mammalian Embryogenesis. Hum. Mol. Genet. 14**,** 1775-1783.
49. Haneline, L. S., Marshall, K. P. and Clapp, D. W. (1996). The Highest Concentration of Primitive Hematopoietic Cells in Cord Blood Is Found in Extremely Premature Infants. Ped. Res. 39**,** 820-825.
50. Hauswirth, W. and Laipis, P. (1985). Transmission Genetics of Mammalian Mitochondria: A Molecular Model and Experimental Evidence in Quagliarello, E. (Ed), Biogenesis, Elsev. Biomed., pp. 49-59.
51. Hauswirth, W. W. and Laipis, P. J. (1982). Mitochondrial DNA Polymorphism in a Maternal Lineage of Holstein Cows. PNAS. 79**,** 4686–4690.
52. Heerdt, B. G. and Augenlicht, L. H. (1990). Changes in the Number of Mitochondrial Genomes During Human Development. Exp.l Cell Res. 186**,** 54-59.
53. Honzik, T., Wenchich, L., Böhm, M., Hansikova, H., Pejznochova, M., Zapadlo, M., Plavka, R. and Zeman, J. (2008). Activities of Respiratory Chain Complexes and Pyruvate Dehydrogenase in Isolated Muscle Mitochondria in Premature Neonates. Early Hum. Dev. 84**,** 269-276.
54. Houstek, J., Andersson, U., Tvrdik, P., Nedergaard, J. and Cannon, B. (1995). The Expression of Subunit C Correlates with and Thus May Limit the Biosynthesis of the Mitochondrial F_0F_1-ATPase in Brown Adipose Tissue. J. Biol. Chem. 270**,** 7689-7694.
55. Huo, L. and Scarpulla, R. C. (2001). Mitochondrial DNA Instability and Peri-Implantation Lethality Associated with Targeted Disruption of Nuclear Respiratory Factor 1 in Mice. Mol. Cell. Biol. 21**,** 644-654.
56. Cha, K.-Y., Lee, S.-H., Chung, H.-M., Baek, K.-H., Cho, S.-W. and Kwack, K.-B. (2005). Retracted: Quantification of Mitochondrial DNA Using Real-Time Polymerase Chain Reaction in Patients with Premature Ovarian Failure. Fert. Steril. 84**,** 1712-1718.
57. Chabi, B., Mousson De Camaret, B., Duborjal, H., Issartel, J. and Stepien, G. (2003). Quantification of Mitochondrial DNA Deletion, Depletion, and Overreplication: Application to Diagnosis. Clin. Chem. 49**,** 1309-1317.
58. Chacinska, A., Pfanner, N. and Meisinger, C. (2002). How Mitochondria Import Hydrophilic and Hydrophobic Proteins. Trends Cell Biol. 12**,** 299-303.
59. Chiu, H. and Phillips, M. (1974). Glucagon Induces Premature Structural Differentiation of Fetal Hepatocytes. Lab. Invest. 30**,** 305-310.
60. Choi, Y.-S., Hong, J.-M., Lim, S., Ko, K. S. and Pak, Y. K. (2006). Impaired Coactivator Activity of the Gly482 Variant of Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1Alpha (PGC-1Alpha) on Mitochondrial Transcription Factor A (TFAM) Promoter. Biochem. Biophys. Res. Comm. 344**,** 708-712.

61. Chow, L., Greenlund, L., Asmann, Y., Short, K., McCrady, S., Levine, J. and Nair, K. (2007). Impact of Endurance Training on Murine Spontaneous Activity, Muscle Mitochondrial DNA Abundance, Gene Transcripts, and Function. J. Appl. Physiol. 102.
62. Izquierdo, J. M., Ricart, J., Ostronoff, L. K., Egea, G. and Cuezva, J. M. (1995). Changing Patterns of Transcriptional and Post-Transcriptional Control of Beta-F_1-ATPase Gene Expression During Mitochondrial Biogenesis in Liver. J. Biol. Chem. 270**,** 10342-10350.
63. Jansen, M. S., Cook, G. A., Song, S. and Park, E. A. (2000). Thyroid Hormone Regulates Carnitine Palmitoyltransferase alpha Gene Expression through Elements in the Promoter and First Intron. J. Biol. Chem. 275**,** 34989-34997.
64. Jauniaux, E., Watson, A. and Burton, G. (2001). Evaluation of Respiratory Gases and Acid-Base Gradients in Human Fetal Fluids and Uteroplacental Tissue between 7 and 16 Weeks' Gestation. Am. J. Obst. Gynecol. 184**,** 998-1003.
65. Jazayeri, M., Andreyev, A., Will, Y., Ward, M., Anderson, C. M. and Clevenger, W. (2003). Inducible Expression of a Dominant Negative DNA Polymerase-Gamma Depletes Mitochondrial DNA and Produces a Rho 0 Phenotype. J. Biol. Chem. 278**,** 9823-9830.
66. Jones, C. and Rolph, T. (1985). Metabolism During Fetal Life: A Functional Assessment of Metabolic Development. Physiol. Rev. 65**,** 357-430.
67. Kaguni, L. S. (2004). DNA Polymerase Gamma, the Mitochondrial Replicase. Ann. Rev. Biochem. 73**,** 293-320.
68. Kang, D., Kim, S. H. and Hamasaki, N. (2007). Mitochondrial Transcription Factor A (TFAM): Roles in Maintenance of Mtdna and Cellular Functions. Mitochondrion Mitochondria and Life 7**,** 39-44.
69. Kelly, D. P. and Scarpulla, R. C. (2004). Transcriptional Regulatory Circuits Controlling Mitochondrial Biogenesis and Function. Gen. Dev. 18**,** 357-368.
70. Kendall, T., Byerley, D. and Dean, R. (1991). Isolation of DNA from Blood. Anal. Biochem. 195**,** 74-76.
71. Kim, K., Lecordier, A. and Bowman, L. (1995). Both Nuclear and Mitochondrial Cytochrome C Oxidase mRNA Levels Increase Dramatically During Mouse Postnatal Development. Biochem. J. 306**,** 353-358.
72. LaMarco, K., Thompson, C., Byers, B., Walton, E. and McKnight, S. (1991). Identification of Ets- and Notch-Related Subunits in Ga Binding Protein. Science 253**,** 789-792.
73. Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S. and Clayton, D. A. (1998). Mitochondrial Transcription Factor A Is Necessary for MtDNA Maintance and Embryogenesis in Mice. Nat. Gen. 18**,** 231-236.
74. Leary, S. C., Battersby, B. J., Hansford, R. G. and Moyes, C. D. (1998). Interactions between Bioenergetics and Mitochondrial Biogenesis. BBA - Bioenerg. 1365**,** 522-530.
75. Legros, F., Malka, F., Frachon, P., Lombes, A. and Rojo, M. (2004). Organization and Dynamics of Human Mitochondrial DNA. J. Cell. Sci. 117**,** 2653-2662.
76. Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M. and Kelly, D. P. (2000). Peroxisome Proliferator Activated Receptor Gamma Coactivator-1 Promotes Cardiac Mitochondrial Biogenesis. J. Clin. Invest. 106**,** 847-856.
77. Lemasters, J. J., Theruvath, T. P., Zhong, Z. and Nieminen, A.-L. (2009). Mitochondrial Calcium and the Permeability Transition in Cell Death. BBA 1787**,** 1395-1401.
78. Lenka, N., Vijayasarathy, C., Mullick, J. and Avadhani, N. (1998). Structural Organization and Transcription Regulation of Nuclear Genes Encoding the Mammalian Cytochrome C Oxidase Complex. Prog. Nucl. Acid Res. Mol. Biol. 61**,** 309-344.
79. Liesa, M., Palacin, M. and Zorzano, A. (2009). Mitochondrial Dynamics in Mammalian Health and Disease. Physiol. Rev. 89**,** 799-845.
80. Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C. and Kispal, G. (1999). The Essential Role of Mitochondria in the Biogenesis of Cellular Iron-Sulfur Proteins. Biol. Chem. 380**,** 1157-1166.

81. Lin, J., Puigserver, P., Donovan, J., Tarr, P., Spiegelman, B.M. (2002). PGC-1beta: a novel PGC-1-related transcription coactivator associated with host cell factor. J. Biol. Chem. 277, 1645–1648.
82. Logan, D. C. (2006). The Mitochondrial Compartment. J. Exp. Bot. 57**,** 1225-1243.
83. Luis, A. M., Izquierdo, J. M., Ostronoff, L. K., Salinas, M., Santarén, J. F. and Cuezva, J. M. (1993). Translational Regulation of Mitochondrial Differentiation in Neonatal Rat Liver. Specific Increase in the Translational Efficiency of the Nuclear-Encoded Mitochondrial Beta-F1-Atpase mRNA. J. Biol. Chem. 268**,** 1868-1875.
84. Masek, T., Vopalensky, V., Suchomelova, P. and Pospisek, M. (2005). Denaturing RNA Electrophoresis in TAE Agarose Gels. Analyt. Biochem. 336**,** 46-50.
85. Masopust, J. (2003). Liver Function Examination in Paediatrics. Pediatrie pro praxi 2**,** 60-62.
86. Matsushima, Y., Garesse, R. and Kaguni, L. S. (2004). Drosophila Mitochondrial Transcription Factor B2 Regulates Mitochondrial DNA Copy Number and Transcription in Schneider Cells. Journal of Biological Chemistry 279**,** 26900-26905.
87. May-Panloup, P., Chretien, M. F., Malthiery, Y. and Reynier, P. (2007). Mitochondrial DNA in the Oocyte and the Developing Embryo. Curr. Top. Dev. Biol. 77:51-83.
88. May-Panloup, P., Vignon, X., Chretien, M.-F., Heyman, Y., Tamassia, M., Malthiery, Y. and Reynier, P. (2005). Increase of Mitochondrial DNA Content and Transcripts in Early Bovine Embryogenesis Associated with Upregulation of MtTFA and NRF1 Transcription Factors. Reprod. Biol. Endocrinol. 3**,** 65.
89. McClure, T. D., Young, M. E., Taegtmeyer, H., Ning, X.-H., Buroker, N. E., Lopez-Guisa, J. and Portman, M. A. (2005). Thyroid Hormone Interacts with PPARAlpha and PGC-1 During Mitochondrial Maturation in Sheep Heart. Am. J. Physiol. Heart Circ. Physiol. 289**,** H2258-2264.
90. Meirhaeghe, A., Crowley, V., Lenaghan, C., Lelliott, C., Green, K., Stewart, A., Hart, K., Schinner, S., Sethi, J. K., Yeo, G., Brand, M. D., Cortright, R. N., O'Rahilly, S., Montague, C. and Vidal-Puig, A. J. (2003). Characterization of the Human, Mouse and Rat PGC1 Beta (Peroxisome-Proliferator-Activated Receptor-Gamma Co-Activator 1 Beta) Gene in Vitro and in Vivo. Biochem. J. 373**,** 155-165.
91. Meller, M., Vadachkoria, S., Luthy, D. A. and Williams, M. A. (2002). Evaluation of Housekeeping Genes in Placental Comparative Expression Studies. Placenta 26**,** 601-607.
92. Miki, A., Mizoguchi, A. and Mizoguti, H. (1988). Histochemical Studies of Enzymes of the Energy Metabolism in Postimplantation Rat Embryos. Histochem. 88**,** 489-495.
93. Miller, F. J., Rosenfeldt, F. L., Zhang, C., Linnane, A. W. and Nagley, P. (2003). Precise Determination of Mitochondrial DNA Copy Number in Human Skeletal and Cardiac Muscle by a PCR-Based Assay: Lack of Change of Copy Number with Age. Nucl. Acids Res. 31**,** e61.
94. Miller, J. (2001). Developmental Biology of Skeletal Muscle in Karpati, G., Hilton-Jones, D. and Griggs, R. (Eds), Disorders of Voluntary Muscle, Cambridge University Press, pp. 26-28.
95. Minai, L., Martinovic, J., Chretien, D., Dumez, F., Razavi, F., Munnich, A. and Rötig, A. (2008). Mitochondrial Respiratory Chain Complex Assembly and Function During Human Fetal Development. Mol. Gen. Metab. 94**,** 120-126.
96. Miyamoto, T., Kaneko, A., Kakizawa, T., Yajima, H., Kamijo, K., Sekine, R., Hiramatsu, K., Nishii, Y., Hashimoto, T. and Hashizume, K. (1997). Inhibition of Peroxisome Proliferator Signaling Pathways by Thyroid Hormone Receptor. J. Biol. Chem. 272**,** 7752-7758.
97. Morten, K. J., Ashley, N., Wijburg, F., Hadzic, N., Parr, J., Jayawant, S., Adams, S., Bindoff, L., Bakker, H. D., Mieli-Vergani, G., Zeviani, M. and Poulton, J. (2007). Liver MtDNA Content Increases During Development: A Comparison of Methods and the Importance of Age- and Tissue-Specific Controls for the Diagnosis of MtDNA Depletion. Mitochondrion 7**,** 386-395.

98. Murphy, N., Hume, R., van Toor, H., Matthews, T. G., Ogston, S. A., Wu, S.-Y., Visser, T. J. and Williams, F. L. R. (2004). The Hypothalamic-Pituitary-Thyroid Axis in Preterm Infants; Changes in the First 24 Hours of Postnatal Life. J. Clin. Endocrinol. Metab. 89**,** 2824-2831.
99. Mutvei, A., Husman, B., Andersson, G. and Nelson, B. (1989). Thyroid Hormone and Not Growth Hormone Is the Principle Regulator of Mammalian Mitochondrial Biogenesis. Acta Endocrinol. (Copenh) 121**,** 223-228.
100. Nicklas, J., Brooks, E., Hunter, T., Single, R. and RF, B. (2004). Development of a Quantitative PCR (Taqman) Assay for Relative Mitochondrial DNA Copy Number and the Common Mitochondrial DNA Deletion in Rat. Env. Mol. Mut. 44**,** 313-320.
101. Nisoli, E., Clementic, E., Moncada, S. and Carruba, M. O. (2004). Mitochondrial Biogenesis as a Cellular Signaling Framework. Biochem. Pharmacol. 67**,** 1-15.
102. Ojala, D., Montoya, J. and Attardi, G. (1981). tRNA Punctuation Model of RNA Processing in Human Mitochondria. Nature 290**,** 470-474.
103. Ongwijitwat, S., Liang, H. L., Graboyes, E. M. and Wong-Riley, M. T. T. (2006). Nuclear Respiratory Factor 2 Senses Changing Cellular Energy Demands and Its Silencing Down-Regulates Cytochrome Oxidase and Other Target Gene mRNAs. Gene 374**,** 39-49.
104. Ongwijitwat, S. and Wong-Riley, M. T. T. (2005). Is Nuclear Respiratory Factor 2 a Master Transcriptional Coordinator for All Ten Nuclear-Encoded Cytochrome C Oxidase Subunits in Neurons ?. Gene 360**,** 65-77.
105. Ostronoff, L. K., Izquierdo, J. M., Enriquez, J. A., Montoya, J. and Cuezva, J. M. (1996). Transient Activation of Mitochondrial Translation Regulates the Expression of the Mitochondrial Genome During Mammalian Mitochondrial Differentiation. Biochem. J. 316**,** 183-191.
106. Padbury, J., ES, E. D., Hobel, C., Perelman, A. and Fisher, D. (1981). Neonatal Adaptation: Sympatho-Adrenal Response to Umbilical Cord Cutting. Pediatr. Res. 15**,** 1483-1487.
107. Papa, S. (1996). Mitochondrial Oxidative Phosphorylation Changes in the Life Span. Molecular Aspects and Physiopathological Implications. BBA 1276**,** 87-105.
108. Parsons, W. J., Williams, R. S., Shelton, J. M., Luo, Y., Kessler, D. J. and Richardson, J. A. (1996). Developmental Regulation of Cytochrome Oxidase Subunit Via Isoforms in Cardiac and Skeletal Muscle. Am. J. Physiol. Heart Circ. Physiol. 270**,** H567-574.
109. Patti, M. E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E. J., Goldfine, A. B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C. R. and Mandarino, L. J. (2003). Coordinated Reduction of Genes of Oxidative Metabolism in Humans with Insulin Resistance and Diabetes: Potential Role of PGC1 and NRF1. PNAS 100**,** 8466-8471.
110. Pejznochova, M., M. Tesarova, Honzik, T., Hansikova, H., Magner, M. and Zeman, J. (2008). The Developmental Changes in Mitochondrial DNA Content Per Cell in Human Cord Blood Leukocytes During Gestation. Physiol. Res. 57**,** 947-955.
111. Pejznochova, M., Tesarova, M., Hansikova, H., Magner, M., Honzik, T., Vinsova, K., Hajkova, Z., Havlickova, V. and Zeman, J. (2010). Mitochondrial DNA Content and Expression of Genes Involved in MtDNA Transcription, Regulation and Maintenance During Human Fetal Development. Mitochondrion Jan 20. [Epub ahead of print].
112. Perez, G. I., Trbovich, A. M., Gosden, R. G. and Tilly, J. L. (2000). Reproductive Biology: Mitochondria and the Death of Oocytes. Nature 403**,** 500-501.
113. Pérez-Novo, C., Claeys, C., Speleman, F., Van Cauwenberge, P., Bachert, C. and Vandesompele, J. (2005). Impact of RNA Quality on Reference Gene Expression Stability. Biotechniques 39**,** 52-56.
114. Persaud, T. and Moore, K. (1998). Introduction to the Developing Human in Persaud, T. and Moore, K. (Eds), The Developing Human, Saunders, pp. 2-3, 396.
115. Philippidis, H. and Ballard, F. (1969). The Development of Gluconeogenesis in Rat Liver: Experiments in Vivo. Biochem. J. 113**,** 651-657.

116. Pollak, J. K. (1977). The Interdependence of Mitochondrial Maturation and Glycogen Metabolism in Perinatal Rat Liver. Biochemi. Soc. Transact. 5**,** 341-348.
117. Poole, A. M. and Penny, D. (2007). Evaluating Hypotheses for the Origin of Eukaryotes. BioEssays 29**,** 74-84.
118. Portman, M. A., Xiao, Y., Qian, K., Tucker, R. L., Parish, S. M. and Ning, X.-H. (2000). Thyroid Hormone Coordinates Respiratory Control Maturation and Adenine Nucleotide Translocator Expression in Heart in Vivo. Circul. 102**,** 1323-1329.
119. Poulton, J., Macaulay, V. and Marchington, D. R. (1998). Is the Bottleneck Cracked ?. Am. J. Hum. Gen. 62**,** 752-757.
120. Prieur, B., Bismuth, J. and Delaval, E. (1998). Effects of Adrenal Steroid Hormones on Mitochondrial Maturation During the Late Fetal Period. European J. Biochem. 252**,** 194-199.
121. Puigserver, P. (2005). Tissue-Specific Regulation of Metabolic Pathways through the Transcriptional Coactivator Pgc1-Alpha. Int. J. Obes. (Lond.) 29**,** S5-S9.
122. Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M. (1998). A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis. Cell 92**,** 829-839.
123. Ramachandran, B., Yu, G. and Gulick, T. (2008). Nuclear Respiratory Factor 1 Controls Myocyte Enhancer Factor 2a Transcription to Provide a Mechanism for Coordinate Expression of Respiratory Chain Subunits. J. Biol. Chem. 283**,** 11935-11946.
124. Reynier, P., May-Panloup, P., Chretien, M.-F., Morgan, C. J., Jean, M., Savagner, F., Barriere, P. and Malthiery, Y. (2001). Mitochondrial DNA Content Affects the Fertilizability of Human Oocytes. Mol. Hum. Reprod. 7**,** 425-429.
125. Ring, J. A., Ghabrial, H., Ching, M. S., Smallwood, R. A. and Morgan, D. J. (1999). Fetal Hepatic Drug Elimination. Pharmacol. Therapeut. 84**,** 429-445.
126. Rizzuto, R., Marchi, S., Bonora, M., Aguiari, P., Bononi, A., Stefani, D. D., Giorgi, C., Leo, S., Rimessi, A., Siviero, R., Zecchini, E. and Pinton, P. (2009). Ca2+ Transfer from the ER to Mitochondria: When, How and Why. BBA 1787**,** 1342-1351.
127. Rodriguez-Cuenca, S., Monjo, M., Gianotti, M., Proenza, A. M. and Roca, P. (2007). Expression of Mitochondrial Biogenesis-Signaling Factors in Brown Adipocytes Is Influenced Specifically by 17beta-Estradiol, Testosterone, and Progesteron. Am. J. Physiol. Endocrinol. Metab. 292**,** E340-346.
128. Rodríguez-Pena, A., Escrivá, H., Handler, A. C. and Vallejo, C. G. (2002). Thyroid Hormone Increases Transcription of Ga-Binding Protein/Nuclear Respiratory Factor-2 alpha-Subunit in Rat Liver. FEBS Letters 514**,** 309-314.
129. Rosmarin, A. G., Resendes, K. K., Yang, Z., McMillan, J. N. and Fleming, S. L. (2004). Ga-Binding Protein Transcription Factor: A Review of Gabp as an Integrator of Intracellular Signaling and Protein–Protein Interactions. Blood Cells Molec. Dis. 32**,** 143-154.
130. Rozen, S. and Skaletsky, H. (2000). Primer 3 on the Www for General Users and for Biologist Programmers. in S, K. and S, M. (Eds), Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana, pp. 365-386.
131. Russell, L. K., Mansfield, C. M., Lehman, J. J., Kovacs, A., Courtois, M., Saffitz, J. E., Medeiros, D. M., Valencik, M. L., McDonald, J. A. and Kelly, D. P. (2004). Cardiac-Specific Induction of the Transcriptional Coactivator Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1Alpha Promotes Mitochondrial Biogenesis and Reversible Cardiomyopathy in a Developmental Stage-Dependent Manner. Circ. Res. 94**,** 525-533.
132. Russo P. (2007). Liver including tumors, gallbladder and biliary tree in Gilbert-Barness E. (Ed), Pathology of the fetus, infant and child, Mosby Elsevier,pp.1207-1210.
133. Sako, Y. (1975). Ultrastructure of the Cardiac Muscle of Early Human Embryo. Jap. Circ. J. 39**,** 1123-1133.
134. Sangawa, H., Himeda, T., Shibata, H. and Higuti, T. (1997). Gene Expression of Subunit C(P1), Subunit C(P2), and Oligomycin Sensitivity-Conferring Protein May Play

a Key Role in Biogenesis of H+-ATP Synthase in Various Rat Tissues. J. Biol. Chem. 272**,** 6034-6037.
135. Sawa, C., Goto, M., Suzuki, F., Watanabe, H., Sawada, J. and Handa, H. (1996). Functional Domains of Transcription Factor hGABP beta1/E4TF1-53 Required for Nuclear Localization and Transcription Activation. Nucl. Acids Res. 24, 4954-4961.
136. Scarpulla, R. C. (2006). Nuclear Control of Respiratory Gene Expression in Mammalian Cells. J. Cell. Biochem. 97**,** 673-683.
137. Scarpulla, R. C. (2008a). Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. Physiol. Rev. 88**,** 611-638.
138. Scarpulla, R. C. (2008b). Nuclear Control of Respiratory Chain Expression by Nuclear Respiratory Factors and PGC-1-Related Coactivator. Ann. New York Acad. Sci. 1147**,** 321-334.
139. Shadel, G. and Clayton, D. (1993). Mitochondrial Transcription Initiation. Variation and Conservation. J. Biol. Chem. 268**,** 16083-16086.
140. Shepard, T. H., Muffley, L. A. and Smith, L. T. (1998). Ultrastructural Study of Mitochondria and Their Cristae in Embryonic Rats and Primate *(N. Nemistrina)*. Anat. Rec. 252**,** 383-392.
141. Shepard, T. H., Tanimura, T. and Park, H. W. (1997). Glucose Absorption and Utilization by Rat Embryos. Int. J Dev. Biol. 41**,** 307-314.
142. Schultz, R. A., Swoap, S. J., McDaniel, L. D., Zhang, B., Koon, E. C., Garry, D. J., Li, K. and Williams, R. S. (1998). Differential Expression of Mitochondrial DNA Replication Factors in Mammalian Tissues J. Biol. Chem. 273**,** 3447-3451.
143. Song, G. J. and Lewis, V. (2008). Mitochondrial DNA Integrity and Copy Number in Sperm from Infertile Men. Fert. Steril. 90**,** 2238-2244.
144. Song, J., Oh, J. Y., Sung, Y.-A., Pak, Y. K., Park, K. S. and Lee, H. K. (2001). Peripheral Blood Mitochondrial DNA Content Is Related to Insulin Sensitivity in Offspring of Type 2 Diabetic Patients. Diabet. Care 24**,** 865-869.
145. Soubannier, V. and McBride, H. M. (2009). Positioning Mitochondrial Plasticity within Cellular Signaling Cascades. BBA1793**,** 154-170.
146. Steuerwald, N., Barritt, J. A., Adler, R., Malter, H., Schimmel, T., Cohen, J. and Brenner, C. A. (2000). Quantification of MtDNA in Single Oocytes, Polar Bodies and Subcellular Components by Real-Time Rapid Cycle Fluorescence Monitored PCR. Zygote 8**,** 209-215.
147. St-Pierre, J., Lin, J., Krauss, S., Tarr, P. T., Yang, R., Newgard, C. B. and Spiegelman, B. M. (2003). Bioenergetic Analysis of Peroxisome Proliferator-Activated Receptor Gamma Coactivators Alpha and Beta (PGC-1alpha and PGC-1beta) in Muscle Cells. J. Biolog. Chem. 278**,** 26597-26603.
148. Stryer, L. (1988). Excitable Membranes and Sensory Systems, Biochemistry, W.H.Freeman and company, pp. 1025-1026.
149. Sutton, R. and Pollak, J. K. (1980). Hormone-Initiated Maturation of Rat Liver Mitochondria after Birth. Biochem. J. 186**,** 361-367.
150. Tamassia, M., Nuttinck, F., May-Panloup, P., Reynier, P., Heyman, Y., Charpigny, G., Stojkovic, M., Hiendleder, S., Renard, J. and Chastan-maillard, S. (2004). In Vitro Embryo Production Efficiency in Cattle and Its Association with Oocyte Adenosine Triphosphate Content, Quantity of Mitochondrial DNA, and Mitochondrial DNA Haplogroup. Biol. Reprod. 71**,** 697-704.
151. Tannirandorn, Y., Uerpairojkit, B., Manotaya, S., Tanawattanacharoen, S., Danthamrongkul, V. and Charoenvidhya, D. (1999). Fetal Hematology. J. Med. Assoc. Thai. 82**,** 598-603.
152. Tata, J., Ernster, L., Lindberg, O., Arrhenius, E., Pedersen, S. and Hedman, R. (1963). The Action of Thyroid Hormones at the Cell Level. Biochem. J. 86**,** 408-428.
153. Tavian, M. and Péault, B. (2005). Embryonic Development of the Human Hematopoietic System. Int. J. Dev. Biol. 49**,** 243-250.

154. Tesarova, M., Mayr, J. A., Wenchich, L., Hansikova, H., Elleder, M., Blahova, K., Sperl, W. and Zeman, J. (2004). Mitochondrial DNA Depletion in Alpers Syndrome. Neuroped. 35**,** 217-223.
155. Truscott, K. N., Brandner, K. and Pfanner, N. (2003). Mechanisms of Protein Import into Mitochondria. Curr. Biol. 13**,** R326-R337.
156. Valcarce, C., Izquierdo, J. M., Chamorro, M. and Cuezva, J. M. (1994). Mammalian Adaptation to Extrauterine Environment: Mitochondrial Functional Impairment Caused by Prematurity. Biochem. J. 303**,** 855-862.
157. Valcarce, C., Navarrete, R., Encabo, P., Loeches, E., Satrustegui, J. and Cuezva, J. (1988). Postnatal Development of Rat Liver Mitochondrial Functions. The Roles of Protein Synthesis and of Adenine Nucleotides. J. Biol. Chem. 263**,** 7767-7775.
158. Vandesompele, J., De Paepe, A. and Speleman, F. (2002). Elimination of Primer-Dimer Artifacts and Genomic Coamplification Using a Two-Step Sybr Green I Real-Time RT-PCR. Analyt. Biochem. 303**,** 95-98.
159. Ventura-Clapier, R., Garnier, A. and Veksler, V. (2008). Transcriptional Control of Mitochondrial Biogenesis: The Central Role of PGC-1Alpha. Cardiovasc. Res. 79**,** 208-217.
160. Virbasius, C. A., Virbasius, J. V. and Scarpulla, R. C. (1993). Nrf-1, an Activator Involved in Nuclear-Mitochondrial Interactions, Utilizes a New DNA-Binding Domain Conserved in a Family of Developmental Regulators. Gen. Dev. 7**,** 2431-2445.
161. Wallace, M. J., Hooper, S. B. and Harding, R. (1995). Effects of Elevated Fetal Cortisol Concentrations on the Volume, Secretion, and Reabsorption of Lung Liquid. Am. J. Physiol. Regul. Integr. Comp. Physiol. 269**,** R881-887.
162. Weitzel, J., Iwen, K. and Seitz, H. (2003). Regulation of Mitochondrial Biogenesis by Thyroid Hormone. Exp. Physiol. 88**,** 121-128.
163. Wenz, T. (2009). PGC-1alpha Activation as a Therapeutic Approach in Mitochondrial Disease. IUBMB Life 61**,** 1051-1062.
164. Wrutniak, C., Cassar-Malek, I., Marchal, S., Rascle, A., Heusser, S., Keller, J.-M., Flechon, J., Daua, M., Samarut, J., Ghysdael, J. and Cabello, G. r. (1995). A 43-KDa Protein Related to c-Erb A 1 Is Located in the Mitochondrial Matrix of Rat Liver. J. Biol. Chem. 270**,** 16347-16354.
165. Wrutniak-Cabello, C., Casas, F. and Cabello, G. (2001). Thyroid Hormone Action in Mitochondria J. Mol. Endocrinol. 26**,** 67-77.
166. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C. and Spiegelman, B. M. (1999). Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. Cell 98**,** 115-124.
167. Wyrsch, A., dalle Carbonare, V., Jansen, W., Chklovskaia, E., Nissen, C., Surbek, D., Holzgreve, W., Tichelli, A. and Wodnar-Filipowicz, A. (1999). Umbilical Cord Blood from Preterm Human Fetuses Is Rich in Committed and Primitive Hematopoietic Progenitors with High Proliferative and Self-Renewal Capacity. Exp. Hematol. 27**,** 1338-1345.
168. Yen, P. M. (2001). Physiological and Molecular Basis of Thyroid Hormone Action. Physiol. Rev. 81**,** 1097-1142.

8 LIST OF ORIGINAL ARTICLES

1) **M.Pejznochova**, M.Tesarova, T.Honzik, H. Hansikova, M.Magner and J.Zeman, The developmental changes in mitochondrial DNA content per cell in human cord blood leukocytes during gestation, Physiological Research, (2008) 57(6):947-955. **IF 1.65**

2) **M. Pejznochova**, M. Tesarova, H. Hansikova, M. Magner, T. Honzik, K. Vinsova, Z. Hajkova, V. Havlickova, J. Zeman, Mitochondrial DNA content and expression of genes involved in mtDNA transcription, regulation and maintenance during human fetal development, Mitochondrion, (2010), in press, doi:10.1016/j.mito.2010.01.006. **IF 4.26**

3) L.Stiburek, D.Fornuskova, L.Wenchich, **M.Pejznochova**, H.Hansikova and J.Zeman, Knockdown of Human Oxa1l Impairs the Biogenesis of F1F0-ATP Synthase and NADH:Ubiquinone Oxidoreductase, Journal of Molecular Biology,(2007), 374(2):506-16. **IF 4.15**

4) T. Honzik, L. Wenchich, M. Böhm, H. Hansikova, **M. Pejznochova**, M. Zapadlo, R. Plavka, and J. Zeman, Activities of respiratory chain complexes and pyruvate dehydrogenase in isolated muscle mitochondria in premature neonates, Early human development, (2008) 84:269-276. **IF 2.12**

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