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Altered gene transcription profiles in fibroblasts harboring either TK2 or DGUOK mutations indicate compensatory mechanisms

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Abstract

Mitochondrial DNA (mtDNA) depletion syndrome (MDS) is an autosomal recessive disorder characterized by a reduced amount of mtDNA, which impairs synthesis of respiratory chain complexes. MDS has been classified into two main groups, the hepatocerebral form affecting liver and the central nervous system, and the myopathic form targeting the skeletal muscle. We have compared the molecular genetic characteristics of fibroblasts derived from two patients harboring *TK2* mutations with two harboring mutations in *DGUOK* gene. Real-time PCR revealed mtDNA depletion in dGK-deficient fibroblasts (dGK$^-$) but not in TK2-deficient cells (TK2$^-$). Real-time RT-PCR and western blotting demonstrated significant differences in the expression of the human equilibrative nucleoside transporter 1 (hENT1) at the mRNA and protein levels. hENT1 transcript and protein were increased in quiescent control and TK2$^-$ fibroblasts relative to cycling cells. In contrast, hENT1 was stable in quiescent and cycling dGK$^-$ cells. Moreover, siRNA down-regulation of hENT1, but not of TK1, induced mtDNA depletion in TK2$^-$ fibroblasts indicating that hENT1 contributes to the maintenance of normal mtDNA levels in cells lacking TK2. Transcripts for thymidine phosphorylase, the mitochondrial transcription factor A (TFAM), and the polymerase gamma (Pol γ), were reduced in dGK$^-$, but not in TK2$^-$ cells while the mRNA expression of thymidylate synthase (TS) increased. Our results suggested differential gene expression in TK2 and dGK-deficient fibroblasts, and highlighted the importance of hENT1 as a compensatory factor in MDS disorder.

Keywords

Fibroblasts; hENT1; mtDNA depletion; Mitochondrial dNTP pools; TK2

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Introduction

Mitochondrial DNA (mtDNA) depletion syndrome (MDS) is a clinically heterogeneous disease characterized by a quantitative reduction of the mtDNA copy number leading to deficiencies of mtDNA-encoded respiratory chain components [1]. MDS has been classified into two main groups, the hepatocerebral form that affects the liver and the central nervous system, and the myopathic form targeting the skeletal muscle. Mutations in eight genes have been causally linked to MDS. Genes involved in the hepatocerebral form include: mitochondrial deoxyguanosine kinase (*DGUOK*) [2], mtDNA polymerase gamma (*POLG1*) [3,4], the human ortholog of the mouse kidney disease gene (*MPV17*) [5], the mitochondrial helicase/primase TWINKLE [6], and the alpha subunit of succinyl-CoA synthase (*SUCLG1*) [7]. Genes responsible of the myopathic form include: mitochondrial thymidine kinase 2 (*TK2*) [8], the β subunit of the succinyl-CoA synthase (*SUCLA2*) [9], and the p53-dependent subunit of ribonucleotide reductase (*RRM2B*) [10].

Synthesis of mtDNA is constitutive and independent of nuclear DNA replication, hence a constant supply of deoxyribonucleoside triphosphates (dNTPs) is crucial for mitochondrial maintenance. In proliferating cells, nuclear dNTPs are produced in the cytosol from ribonucleotides by the *de novo* pathway via the key enzymes ribonucleotide reductase and thymidylate synthase, and by the phosphorylation of deoxyribonucleosides by two salvage enzymes, thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK). Mitochondrial dNTPs can be imported directly from the cytosol by dedicated transporters [11,12]. In non-replicating cells with low levels of *de novo* synthesis and TK1 activity, salvage of deoxyribonucleosides by the intra-mitochondrial enzymes deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2) is the major source of mt-dNTP pools. Nevertheless, quiescent cells perform *de novo* synthesis, albeit at very low levels compared to cycling cells [13].

Although TK2 and dGK are constitutively expressed and carry out analogous functions catalyzing the first step in the mitochondrial nucleotide *salvage* pathway, the clinical features of the patients are clearly different [2,8]. It remains unclear why the *TK2* mutations mainly cause myopathy and *DGUOK* mutations predominantly affect liver. One possible explanation is that in unaffected tissues either activity may be rescued by cytoplasmic enzymes with overlapping substrate specificity. This explanation is plausible for dGK deficiency because cytoplasmic dCK activity is very low in liver and brain rendering these tissues particularly vulnerable to this deficiency [14]. Regarding TK2 deficiency, low basal TK2 activity and high metabolic demands of skeletal muscle may contribute to the selective vulnerability of this tissue [15]. Tissue specificity is also reflected in cultured fibroblasts derived from TK2 and dGK-deficient patients. To our knowledge, only fibroblasts derived from patients harboring mutations in *DGUOK* gene have shown mtDNA depletion [16–20] while TK2-deficient fibroblasts have had normal levels of mtDNA [21,22]. To elucidate molecular mechanisms, which may account for the differences in mtDNA levels that are probably related to the intrinsic regulation of nucleoside/nucleotide metabolism, we have compared the molecular genetic profiles of two TK2 and two dGK-deficient fibroblasts. We found marked differences in the expression of human equilibrative nucleoside transporter 1 (hENT1) and TK1 at the mRNA and protein levels. Moreover, down-regulation of hENT1,

but not TK1, induced mtDNA depletion in TK2-deficient fibroblasts. Our results suggested differential gene expression profiles in TK2 and dGK-deficient fibroblasts, and highlight the importance of hENT1 in the expression of MDS disorder.

Materials and methods

Patients

Patient 1 (P1 TK2⁻)—A 16-year-old patient affected by a severe myopathy, harboring two allelic mutations in the TK2 gene, R152G and K171Δ (new annotation R225G and K244Δ). The patient's muscle showed a marked decrease in TK2 activity, impairment of the respiratory chain complexes, but normal levels of mtDNA [23].

Patient 2 (P2 TK2⁻)—A 12-year-old patient also affected by the myopathic form of MDS, harbored two allelic mutations in the TK2 gene, T77 M and R161 K (new annotation T150 M and R234 K [24]). He developed severe muscle atrophy with fat infiltration, 95% reduction of mtDNA in muscle, but normal activity of the respiratory chain complexes [25,26].

Patient 3 (P1 dGK⁻)—This individual, harboring a homozygous mutation in the *DGUOK* gene (L250 S), suffered from early-onset rapidly progressive and lethal liver failure associated with severe mtDNA depletion in liver [25].

Patient 4 (P2 dGK⁻)—This patient died at early childhood. He had a duplication of GATT sequence at nucleotides 848–851 and a E165 V mutation in the *DGUOK* gene. Muscle had a normal amount of mtDNA.

Cell lines and cell growth

Human skin fibroblasts cell lines derived from patients 1–4 and from 3 control donors were studied. For simplicity, we will refer them as TK2⁻, dGK⁻, and control cell lines, specifying P1 or P2 when required. Cells where grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g of glucose/liter supplemented with 10% dialyzed fetal bovine serum (dFBS), 1% L-glutamine, 1% non-essential amino acids, 100 U/ml antibiotic/antimycotic, and 50 μg/ml of uridine. For most of the experiments, cells have been grown at pre-confluence (cycling cells) or at 5, 10, 15, or 20 days of post-confluence. To maintain quiescent cultures after cells reached confluency, we changed the culture medium to 0.1% dFBS with DMEM and maintained the cells for 20 days adding fresh medium every day. We used flow cytometry (FACS Calibur™ System. BD Biosciences) to determine cell-cycle distribution. Cells were regularly tested for mycoplasma contamination using the Myco-Alert Mycoplasma Detection kit (Lonza).

Quantification of mtDNA depletion

DNA content assays were performed with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Total DNA was extracted from cultured cells. mtDNA was measured using the 12 S ribosomal TaqMan mitochondrial assay labeled with 6FAM fluorochrome and the primers 5′-CCACGGGAAACAGCAGTGATT-3′ and 5′-

CTATTGACTTGGGTTAATCGTGTGA-3′. Measurements of the nuclear single copy gene *RNAseP* was performed by using the kit PDARs RNAseP (Applied Biosystems) and a VIC labeled probe. We used calibration curves to quantitate the number of mtDNA versus nDNA copies.

Anti-TK2 antibody synthesis

The specific TK2 synthetic peptide H-RRAWPPDKEQEKEKKS-NH_2 was coupled to keyhole limpet hemocyanin (KLH). Three *New Zealand* rabbits were injected with 200 μg of the synthetic peptide conjugate in MPL-TDM adjuvant (Sigma) every 2 weeks, and after four immunizations, animals were boosted monthly. The rabbits were bled from the auricular artery, 7 days after each immunization starting from the third dose. Serum samples were stored frozen at −40 °C until used. Animal protocols were approved by the CEEA (Ethical Committee of Animal Experimentation) of our Institution. Pre-immune rabbit sera were used as negative control.

Immunoblotting

Cells grown at different culture conditions were collected and cell protein lysates prepared by adding protease inhibitor cocktail (Sigma) to the lysis buffer (0.1 M Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EGTA,1 mM EDTA,1% Triton X-100), and quantified by Lowry colorimetric assay (RC DC, BioRad Laboratories) with bovine serum albumin as standard. Gel electrophoresis was performed with 0.1% SDS and 15% polyacrylamide and transferred to polyvinylidine difluoride (PVDF) membranes (Westran® S, Whatman). The TK2 polyclonal antibody was tested using kidney and liver protein lysates as tissues with high content in mitochondria. A range of 10–50 μg of protein was loaded and tested with the antibody diluted 1/500 to 1/5000. A 26 kDa band was detected. To demonstrate specificity of the signal, we performed inhibition assays with the TK2 peptide (not shown). The same procedure was followed with control fibroblasts cells. The optimal conditions were detected for 30 μg of protein using 1/3000 dilution of the anti-serum. Three million fibroblasts obtained from a confluent Petri dish (10 cm of diameter) allowed us to obtain approximately 300 μg of total protein which was enough to perform several blots. The antibody against the human isoform of ENT1 was a kind gift from Prof. M. Pastor from the University of Barcelona. Mouse monoclonal COXII antibody was purchased from Molecular Probes (A-6404, Invitrogen). All secondary antibodies were anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated (Dako). Blots were developed with enhanced chemiluminescence (ECL) ECL-Plus Kit (GE Healthcare). Same blots were re-probed with 1/3000 mouse monoclonal anti-VDAC (Calbiochem Anti-Porin 31HL) to normalize the amount of mitochondrial protein loaded. Densitometric analyses were performed quantifying non-saturated autoradiographs with Quantity One v4.0.3 analysis software (BioRad Laboratories).

mRNA expression analysis

Quantitative RT-PCR. mRNA expression assays were performed with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Total RNA was obtained from cells (Tri-Pure, Roche), treated with RNase-free DNase, and quantified by spectrophotometry. Reverse transcription (RT) was performed using the High-Capacity

cDNA Archive Kit (Applied Biosystems) and PCR was performed using Gene Expression Assays Taqman® probes for: thymidine kinase 1 (TK1) (Hs00177406_m1), mitochondrial deoxyguanosine kinase (*DGUOK*) (Hs00361549_m1), deoxycytidine kinase (*DCK*) (Hs00176127_m1), human equilibrative nucleoside transporter 1 (*hENT1*) (SLC29A1) (Hs00191940_m1), adenine nucleotide translocator 1 (*ANT1*) (Hs00154037_m1), mitochondrial DNA polymerase gamma (*POLG*) (Hs00160298_m1), thymidylate synthase (TS) (TYMS) (Hs00426591_m1), thymidine phosphorylase (TP) (*TYMP*) (Hs00157317_m1), mitochondrial transcription factor A (*TFAM*) (Hs00273372_s1), mitochondrial transcription factor B1 (*TFB1 M*) (Hs00274971_m1), mitochondrial transcription factor B2 (*TFB2 M*) (Hs00254651_m1), and the endogen control cyclophilin A (*PPIA*) (Hs99999904_m1). PCR was performed following cycle conditions: an initial step of 50 °C for 2 min, then 95 °C for 10 min, and 40 repetitions of two-step cycles of 50 °C for 15 s and 60 °C for 1 min. All assays were performed at least in triplicate. Post-PCR analysis was analyzed by using GenAmp 7500 SDS software.

TK1 enzyme assay

Cycling (pre-confluence) and quiescent cells (10-day post-confluence in 0.1% dFBS) were collected. The TK1 whole cell extracts were lysed in 50 mM Tris–HCl (pH 7.6) 2 mM DTT, 5 mM benzamidine, 5% NP-40, 2.5% glycerol, and 0.5 mM PMSF. The TK1 activity was determined by radiochemical method using the DE-81 filter paper technique as previously described [27]. Each reaction was carried out with 80 μg of whole extract in a 100 μl final volume of 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, and 10 mM NaF. The substrate for TK activity was 1 μM 3^H-thymidine and the inhibitor for TK2 was 500 μM cold cytidine.

Knockdown of TK1 and hENT1

Control, $TK2^-$, and dGK^- fibroblasts were infected with lentiviral particles containing a U6 promoter driven by a shRNA targeting TK1 and hENT1. These targeting construct belongs to the MISSION TCR-Hs 1.0 shRNA library (NM_003258 for *TK1* and NM_004955 for *hENT1* transcripts) (Sigma-Aldrich). The specific hairpin sequences are comprised of a 21-base stem and 6-base loop and each cloned into pLKO.1 vector. Five shRNA constructs for each target gene are created by Sigma to provide varying levels of knockdown and to target different regions of mRNA transcript. In the initial experiments, we tested all 5 constructs for each gene to validate the best shRNA construct to interfere. Infective particles were generated in HEK293T cells by co-transfection of specific shRNA together with VSVG, RTR2, and PKGPIR vectors, which provide the envelope, packaging and reverse-expressing proteins, respectively (Sigma-Aldrich). Supernatant of these cells were taken at 48 and 72 h post-transfection, filtered, supplemented with fresh dFBS and non-essential amino acids, and used to infect control fibroblasts. The generation of the infective particles and the infection of fibroblasts are performed at 33 °C. Two days later, infected cells were selected with puromycin at 1 μg/ml. Ten days later, cells were processed to test the level of TK1 and hENT1 mRNAs by real-time RT-PCR as we have described above. Two constructs were selected, 44 for hENT1 and 135 for TK1, which were use in consecutive experiments in control, $TK2^-$, and dGK^- cells following the same procedure. Cells grown in 10% dFBS were infected with lentiviral particles containing specific hENT1, TK1, or control (shCV)

shRNAs. After 2 days, medium was shifted to 0.1% dFBS, and puromycin added. Cells were harvested 10 days later, DNA and RNA extracted and assayed for mtDNA depletion using real-time PCR as described above. In parallel, the efficiency of the assay was verified measuring the mRNA expression of hENT1 and TK1 by real-time RT-PCR (results not shown). For this experiment we used 3 different control fibroblasts from healthy volunteers grouped as mean controls for each shRNA.

Results

Depleting cells of dFBS induced mtDNA depletion in dGK⁻ fibroblasts, but not in TK2⁻ fibroblasts

Fibroblasts from a healthy volunteer control and from patients 1–4 (P1 TK2⁻, P2 TK2⁻, P1 dGK⁻ and P2 dGK⁻) were grown to confluence in 10% dFBS medium and then shifted or not to 0.1% dFBS for 5, 10, 15 and 20 days. In these culture conditions, high proportions of cells became quiescent, simulating tissues involved in MDS, which predominantly affects resting cells. Flow cytometry analyses showed that fibroblasts grown at 10-day post-confluence in 0.1% of dFBS were >97% in G1/G0 (results not shown). Considering confluence as day 0, from day 1 of post-confluence, medium was changed daily to maintain attachment of the cell monolayer. Using a quantitative-PCR-based system to assess the relative amount of mtDNA respect to nuclear DNA (nDNA) in each culture condition, levels of mtDNA showed no mtDNA depletion in control and in TK2⁻ fibroblasts for several weeks (Figs. 1A and B). Furthermore, in 0.1% dFBS at day 20, the amount of mtDNA increased in both, control and TK2⁻ cells. The basal amount of mtDNA in TK2⁻ cells cultured in 10% dFBS was higher than in control cells. Similar results were observed in control and TK2-deficient myoblasts when induced to differentiate to myotubes during 40 days (not shown). This was probably due to a cellular response to stress conditions when cell-cycle arrest was induced. In contrast, dGK⁻ fibroblasts cultured in dFBS for 20 days showed a decline of mtDNA abundance (58% residual mtDNA in P1 dGK⁻ and 67% in P2 dGK⁻ relative to pre-confluence cells) (Fig. 1C). Because TK2 and dGK are two enzymes involved in the parallel mitochondrial pathways, these results indicated that different molecular responses occur when we induced the mtDNA depletion phenotype in TK2 and dGK-deficient fibroblasts by serum starvation.

TK2 protein expression was diminished in TK2⁻ but not in dGK⁻ fibroblasts

TK2 activity in fibroblasts of these two patients has been reported [23,25], but not expression of the protein. By western blotting with our TK2 anti-serum, we detected a 26 kDa protein, which was markedly reduced in TK2-deficient fibroblasts compared to control (C) or dGK⁻ fibroblasts (Fig. 2) indicating that the mutations caused degradation of the protein. Moreover, peptide inhibition immunoblot confirmed specificity of the TK2 antibody (results not shown). Anti-porin (VDAC) antibody was blotted in the same membrane to normalize the amount of mitochondrial protein loaded onto each lane.

hENT1 expression differed between TK2⁻ and dGK⁻ quiescent fibroblasts

Given the differences in the mtDNA depletion phenotype exhibited by TK2⁻ and dGK⁻ fibroblasts, we proceeded to measure expression of the equilibrative nucleoside transporter 1

(hENT1), which is localized in the mitochondrial inner membrane (MIM) [28,29] and plasma membrane of the cell, and transports purine and pyrimidine nucleosides into the mitochondrial matrix. mRNA expression was measured by real-time RT-PCR using specific Taqman® probes. Cycling cells of each fibroblast cell line were used as a calibrator (RQ value 1), to compare quiescent (d10) to cycling cells (Pr) of each line (Fig. 3A). Thus, when control fibroblasts were cultured with 0.1% dFBS for 10 days after confluence, hENT1 mRNA was up-regulated compared to the expression found in cycling cells ($p< 0.05$). TK2$^-$ fibroblasts showed the same response, but dGK$^-$ cells maintained hENT1 mRNA levels that were similar to those of cycling cells (Fig. 3A). To assess the relative amount of up-regulation of hENT1 among quiescent cells, we expressed the same results using the level of ENT1 mRNA in quiescent control fibroblasts as a calibrator (value=1) (Fig. 3B). Even though, expression of hENT1 in TK2$^-$ quiescent fibroblasts was significantly higher than in dGK$^-$ cells, indicating that in quiescence hENT1 mRNA is lower in patients than in control fibroblasts, with dramatically down-regulation of the transcript in dGK$^-$ cells. The hENT1 protein followed the same expression pattern as the mRNA, with increased levels in control and TK2$^-$ quiescent fibroblasts but not in dGK$^-$ cells (Fig. 3C). Because this transporter imports nucleosides into mitochondria, these results reveal a potentially important mechanism that may account for the absence of mtDNA depletion in TK2$^-$ fibroblasts.

TK1 mRNA expression, but not activity, is increased in quiescent TK2$^-$ fibroblasts

Because cytosolic thymidine kinase 1 (TK1) expression is tightly regulated and cell-cycle dependent, we screened for altered TK1 transcript expression in TK2$^-$ and dGK$^-$ fibroblasts. We observed a significant increase in TK1 mRNA expression in quiescent TK2$^-$ fibroblasts relative to quiescent control and dGK$^-$ cells ($p<0.05$), which showed no significant changes (Fig. 4A). The lack of mtDNA depletion phenotype and increased expression of TK1 transcript in quiescent TK2-deficient cells suggested that TK1 activity would also be increased; however, enzymatic activity of TK1 was dramatically decreased in quiescent control cells (5%) and TK2$^-$ fibroblasts (1% in both P1 and P2) relative to dividing cells (Fig. 4B). These results suggested that TK1 activity cannot account for the lack of mtDNA depletion in TK2-deficient fibroblasts.

Down-regulation of hENT1, but not TK1, induced mtDNA depletion in TK2$^-$ fibroblasts

We studied the impact of knocking down hENT1 and TK1 transcripts on the relative mtDNA abundance of control, TK2$^-$, and dGK$^-$ fibroblasts using a lentiviral system harboring antisense small hairpins (shRNA). TK2$^-$ cells infected with control vector (shCV) or TK1 shRNA showed increased levels of mtDNA, relative to control fibroblasts (Fig. 5A). However, a significant reduction in the mtDNA was observed in TK2$^-$ cells transfected with hENT1 shRNAs ($p<0.05$) not only relative to shCV transfected TK2$^-$ cells, but also compared to hENT1 shRNA transfected control cells. Cells infected with shCV showed an increase in the mtDNA content relative to control cells, also probably due to a cellular response to stress conditions when cell-cycle arrest was induced (Figs. 1A and B). Fig. 5B shows the results of the analogous experiments performed in dGK$^-$ fibroblasts. Unlike TK2$^-$ cells, the amount of mtDNA in dGK$^-$ cells was reduced in both, hENT1 and TK1 shRNA transfected cells relative to the corresponding control cells ($p<0.001$ for hENT1$^-$ and $p<0.05$ for TK1$^-$) (Fig. 5B). Cells infected with shCV also showed a small reduction in the mtDNA

relative to control cells ($p < 0.05$) (Fig. 5B). These results demonstrate the possibility of inducing mtDNA depletion by abrogation of hENT1 in TK2-deficient fibroblasts.

Expression of genes involved in the nucleoside/nucleotide metabolism and mtDNA maintenance

Next, we analyzed the mRNA expression levels of other genes that could contribute to the different phenotypes of quiescent TK2$^-$ and dGK$^-$ fibroblasts by real-time RT-PCR. The genes analyzed are involved in dNTP pool homeostasis, mtDNA transcription and replication, and nucleoside/nucleotide transport across the inner mitochondrial membrane. The mRNA levels of thymidylate synthase (TS), which is an enzyme that maintains the deoxythymidine monophosphate (dTMP) pool by converting deoxyuridine monophosphate (dUMP) to dTMP, were decreased in TK2$^-$, but not in dGK$^-$ fibroblasts compared to control cells ($p<0.05$) (Table 1). Thymidine phosphorylase (TP) which catabolyzes deoxythymidine to thymine and deoxyuridine to uracil, showed the opposite behavior, with significant decreases of mRNA levels in dGK$^-$ but not in TK2$^-$ cells compared to control cells ($p<0.05$ for P1 and $p<0.001$ for P2). Both, mitochondrial transcription factor A (TFAM) and Pol γ are two proteins strongly associated with the maintenance of mtDNA. TFAM is an essential mtDNA transcription factor that is required for transcribing mtDNA-encoded proteins and also for initiating the mtDNA replication, and Pol γ is the mitochondrial polymerase necessary to replicate mtDNA. Both genes were reduced in dGK$^-$ quiescent fibroblasts ($p<0.05$), but not in TK2-deficient cells (Table 1). No significant alterations of mRNA expression of the *salvage* kinases, DCK and DGUOK, the mitochondrial 5′ nucleotidase dNT2, the mitochondrial deoxynucleotide kinases nm23-H4 and nm23-H6, the transporter ANT1, and the mitochondrial transcription factors TFB1 M and TFB2 M, between TK2$^-$ and dGK$^-$ fibroblasts (Table 1).

Discussion

Maintenance of mtDNA is controlled by a complex homeostatic network, whose effectors include various components of the mtDNA replisome, and multiple enzymes and carrier proteins that provide mitochondria with a balanced supply of deoxyribonucleotide precursors. Alterations of these components can cause mtDNA depletion. To date, mutations in 8 genes have been described as primary causes of this syndrome [2,3,5–10]. In this paper, we have focused on possible interrelationships of TK2 and dGK salvage mitochondrial enzymes with other proteins also involved in the maintenance of this tightly controlled homeostatic network. Fibroblasts derived from well-characterized TK2 and dGK-deficient patients [23,25,26] showed differences in mtDNA levels; dGK$^-$ quiescent fibroblasts showed mtDNA depletion, while TK2$^-$ cells maintained normal mtDNA copy numbers. These results are consistent with published works [16–22]. We also showed that TK2-deficient fibroblasts expressed low levels of TK2 protein, suggesting that not only activity of the enzyme, but also synthesis or stability of the protein is compromised in these cells.

These two first observations prompted us to analyze how the TK2 or dGK deficiencies could affect the expression of other genes involved in the maintenance of the mtDNA and the regulation of nucleoside/nucleotide homeostasis that could account for absence of mtDNA

depletion in TK2^{-} fibroblasts. Metabolic pathways that provide mitochondrial dTTP in cultured cells have been characterized [30]. In cycling cells, R1/R2 ribonucleotide reductase and, to a lesser extent, TK1 were the major sources of the nucleotide. dTMP enters mitochondria [12] by an unknown transporter and is phosphorylated to dTTP by intra-mitochondrial kinases. By contrast, in quiescent cells, dTMP is synthesized by the R1/p53R2 ribonucleotide reductase in the cytosol [13,31] and by salvage of thymidine by TK2 in mitochondria [32]. Although the mitochondrial inner membrane is impermeable to nucleotides, cytosolic and mitochondrial dNTP pools are in rapid communication [30,33], highlighting the importance of specific transporters [11,12].

We observed significant differences in the expression of human equilibrative nucleoside transporter (hENT1) between TK2 and dGK-deficient fibroblasts. hENT1 transports a wide range of purine and pyrimidine nucleosides and nucleobases, is present almost ubiquitously in human tissues [34], and is located in both, plasma and mitochondrial membranes [28,29]. Ours results led us to hypothesize that hENT1 plays an important role in preventing mtDNA depletion in TK2-deficient fibroblasts. hENT1 mRNA and protein expression clearly differed in TK2 and dGK-deficient fibroblasts, with a marked decreases of both in dGK^{-} quiescent cells. Decreases of hENT1, in to the setting of reduced activity of dGK enzyme due to DGUOK mutations, and probably the imbalanced intra-mitochondrial dGTP pools, as reported by A. Saada in one dGK^{-} patient [20], accounts for the mtDNA depletion phenotype exhibited by dGK^{-} cells. The fact that abrogation of hENT1 mRNA in TK2^{-} quiescent fibroblasts induced mtDNA depletion (Fig. 5), and the unchanged mitochondrial and cytosolic dTTP pools in our TK2^{-} patients reported by Frangini et al. [35], strongly supports this hypothesis and demonstrated a remarkable difference between the two genetic backgrounds.

In quiescent TK2^{-} fibroblasts, we observed a significant increase in TK1 transcript, which was not accompanied by an increase in its activity (Fig. 4). This is not surprising given the loose correlation between mRNA levels and enzyme activities observed in some tissues [36]. Thymidylate synthase (TS) catalyzes the conversion of dUMP to dTMP. Pontarin et al. [13] have demonstrated the existence of a complete *de novo* pathway of pyrimidine deoxyribonucleotide synthesis in cultured quiescent fibroblasts to provide deoxynucleotides for mitochondrial DNA replication and low R1/p53R2 ribonucleotide reductase activity correlates with low TS activity [13,31]. We found TS mRNA expression down-regulated in TK2^{-} quiescent fibroblasts probably because the synthesis of dTMP mainly occurs through R1/p53R2 ribonucleotide reductase [13,31]. Thus, *de novo* synthesis of dTTP together with the high expression of ENT1 could compensate for TK2 deficiency and account for the normal mtDNA levels in the TK2^{-} quiescent fibroblasts. Levels of TFAM and Pol γ transcripts were normal in TK2^{-} fibroblasts, but were decreased in dGK-deficient cells, which had less mtDNA. Thus, TFAM and Pol γ were probably decreased due to the reduced requirements of these two proteins in the setting of mtDNA depletion. These results agree with the direct correlation found between mtDNA and TFAM levels [37,38] and are consistent with the observation that TFAM plays an important role in packaging and stabilizing mtDNA by coating the entire molecule (reviewed in [39]). The Pol γ decrease in dGK-deficient fibroblasts was probably a response to low levels of substrates for the replication of the mtDNA.

Conclusions

Taken together, our results demonstrated that these fibroblast cell lines, harboring different mutations, had dissimilar gene expression patterns that alters the complex equilibrium necessary for the proper synthesis of mtDNA and probably define different molecular mechanisms or varying compensatory capacities for the low mtDNA amount *in vivo*. These observations shed light on the molecular mechanisms underlying the cell-specificity of the MDS disorder.

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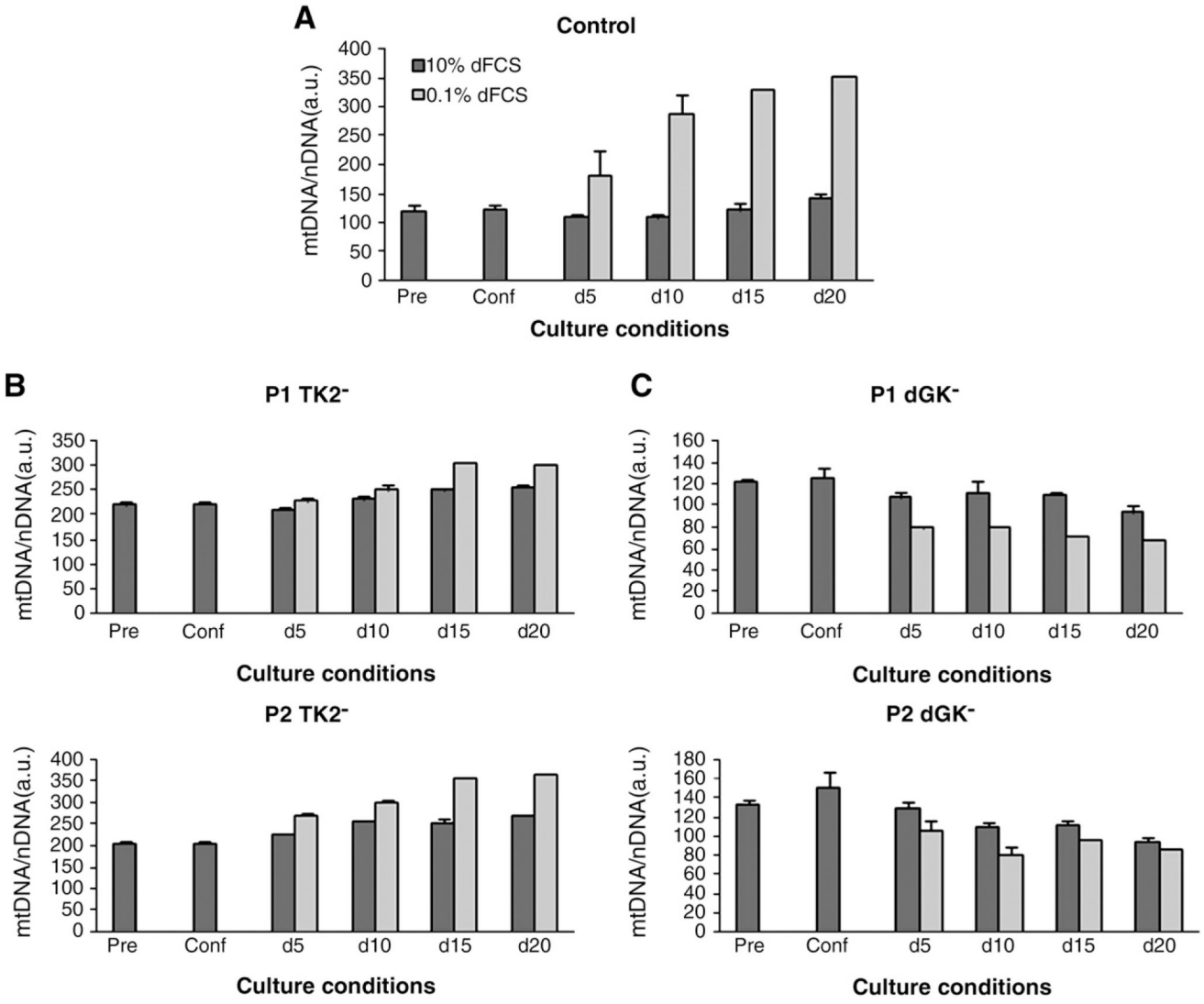

Fig. 1 –.

Measurement of mtDNA depletion by quantitative real-time PCR. Control (A), TK2 (B) and dGK-deficient fibroblasts (C) were grown in 10% dFBS (dark bars) until reaching confluence and then grown in 0.1% dFBS (grey bars) or 10% FBS for 5–20 days post-confluency: day 5 (d5), 10 (d10), 15 (d15), and 20 (d20). The ratio between mitochondrial and nuclear DNA are expressed in arbitrary units (a.u). Pre (cycling cells), Conf (confluence cells). Dark bars, 10% dFBS; grey bars, 0.1% dFBS.

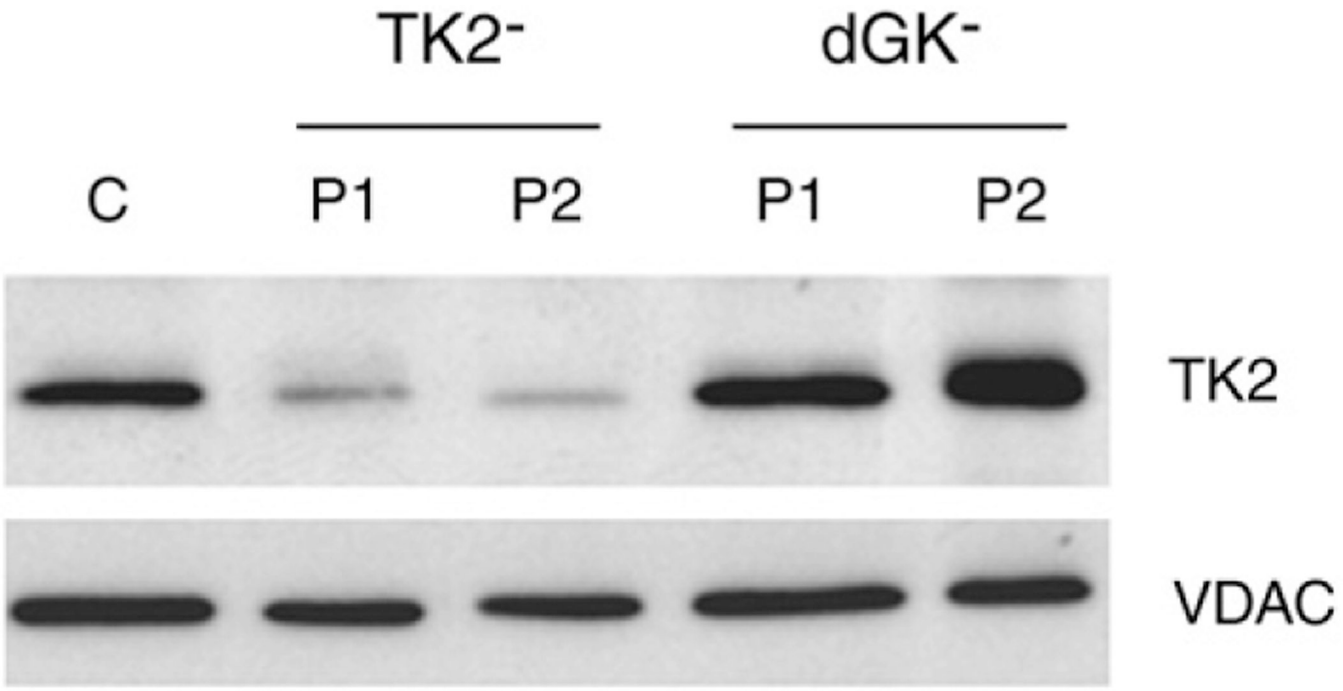

Fig. 2 –.

Expression of TK2 protein in fibroblasts determined by Western blotting. Control (C), TK2, and dGK-deficient fibroblasts were grown until confluence in 10% dFBS. We blotted 20 μg of cell protein with TK2 anti-serum diluted 1:3000 in blocking buffer. A 26 kDa band corresponding to TK2 protein was detected. The membrane was stripped in the appropriate buffer and re-blotted with anti-porin antibody (VDAC) to normalize the amount of protein loaded onto each lane.

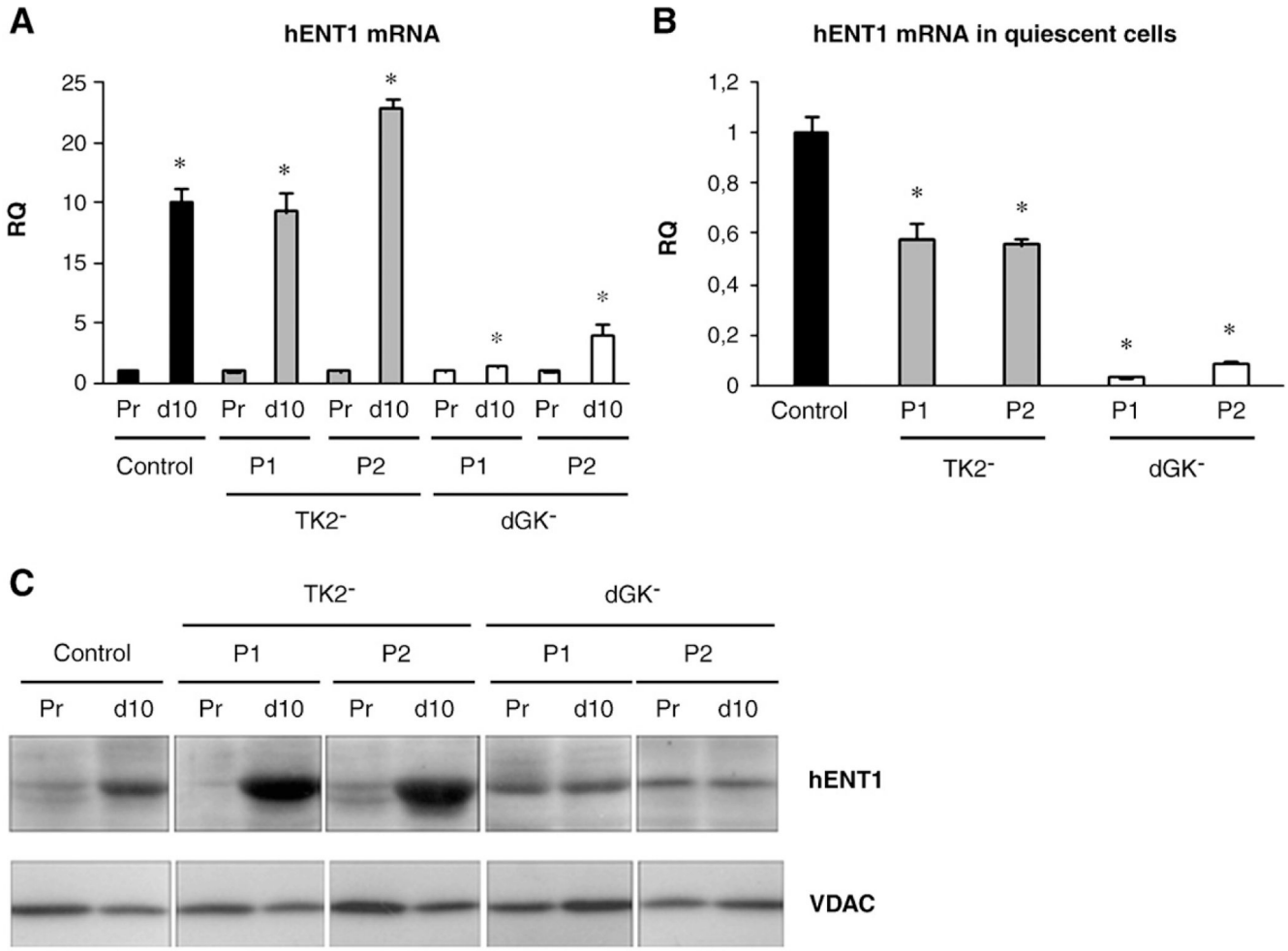

Fig. 3 –.

Study of the expression of hENT1 at mRNA and protein levels in control and patient's fibroblasts. (A) mRNA expression of hENT1 in cycling (Pr) and quiescent cells (d10) by real-time RT-PCR. Transcript levels in quiescent cells of each patient were normalized to baseline transcript levels in cycling cells. Cyclophilin A was used as an endogenous control gene. RQ: relative quantity. (B) Same experiment as in A, but comparing only cells in quiescence. Control quiescent fibroblast was then taken as a calibrator. (*) means $p<0.05$. (C) WB showing the expression of hENT1 protein in cycling (Pr) and quiescent fibroblasts (d10) in control, $TK2^{-}$ and dGK^{-} fibroblasts. Anti-porin (VDAC) antibody was blotted in the same membrane to normalize the amount of mitochondrial protein loaded.

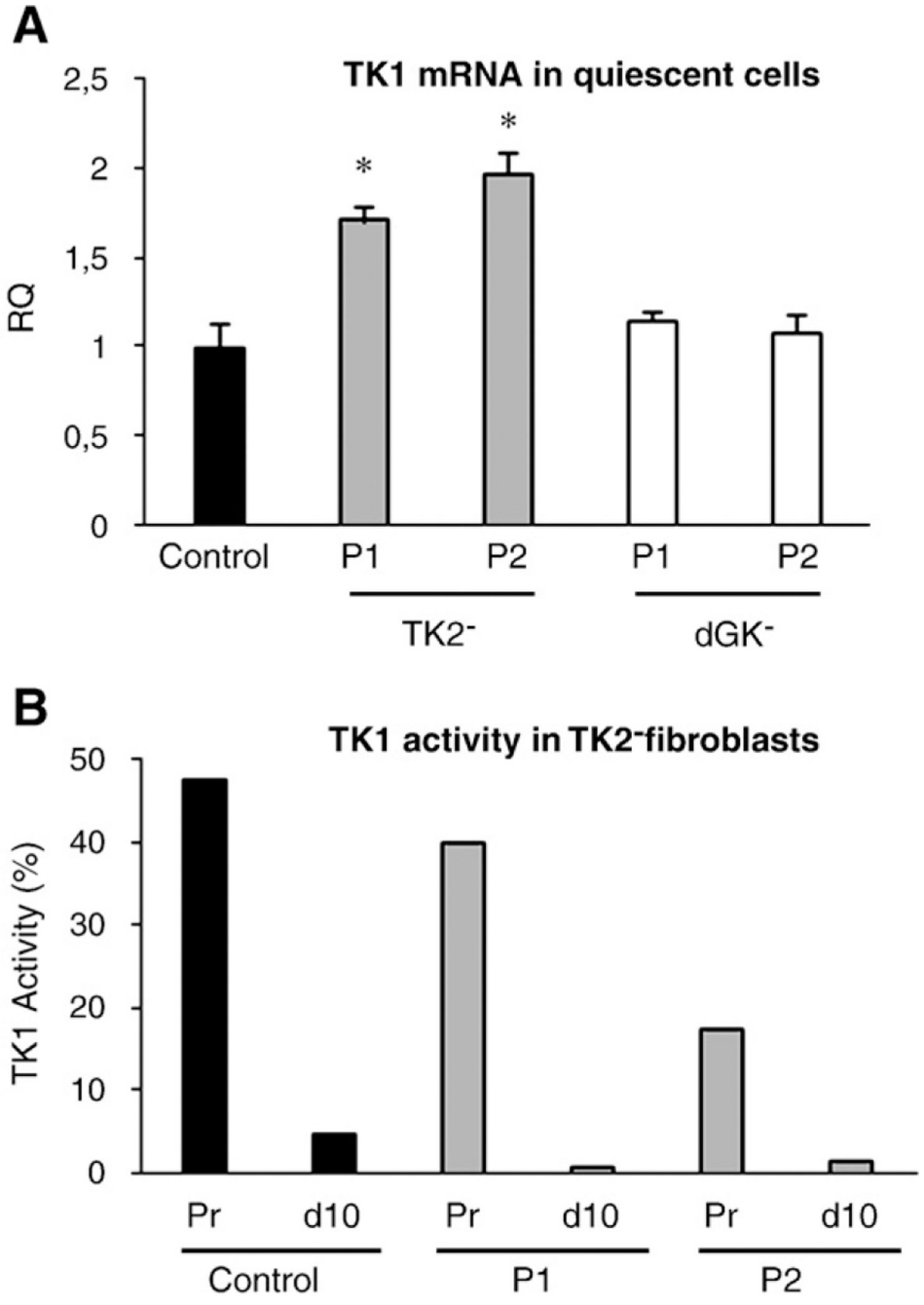

Fig. 4 –.

Analysis of mRNA expression and activity of TK1. mRNA expression of TK1 in quiescent fibroblasts by real-time RT-PCR (A). TK1 activity expressed in percentage (%) in cycling (Pr) and quiescent (d10) TK2-deficient fibroblasts (B).

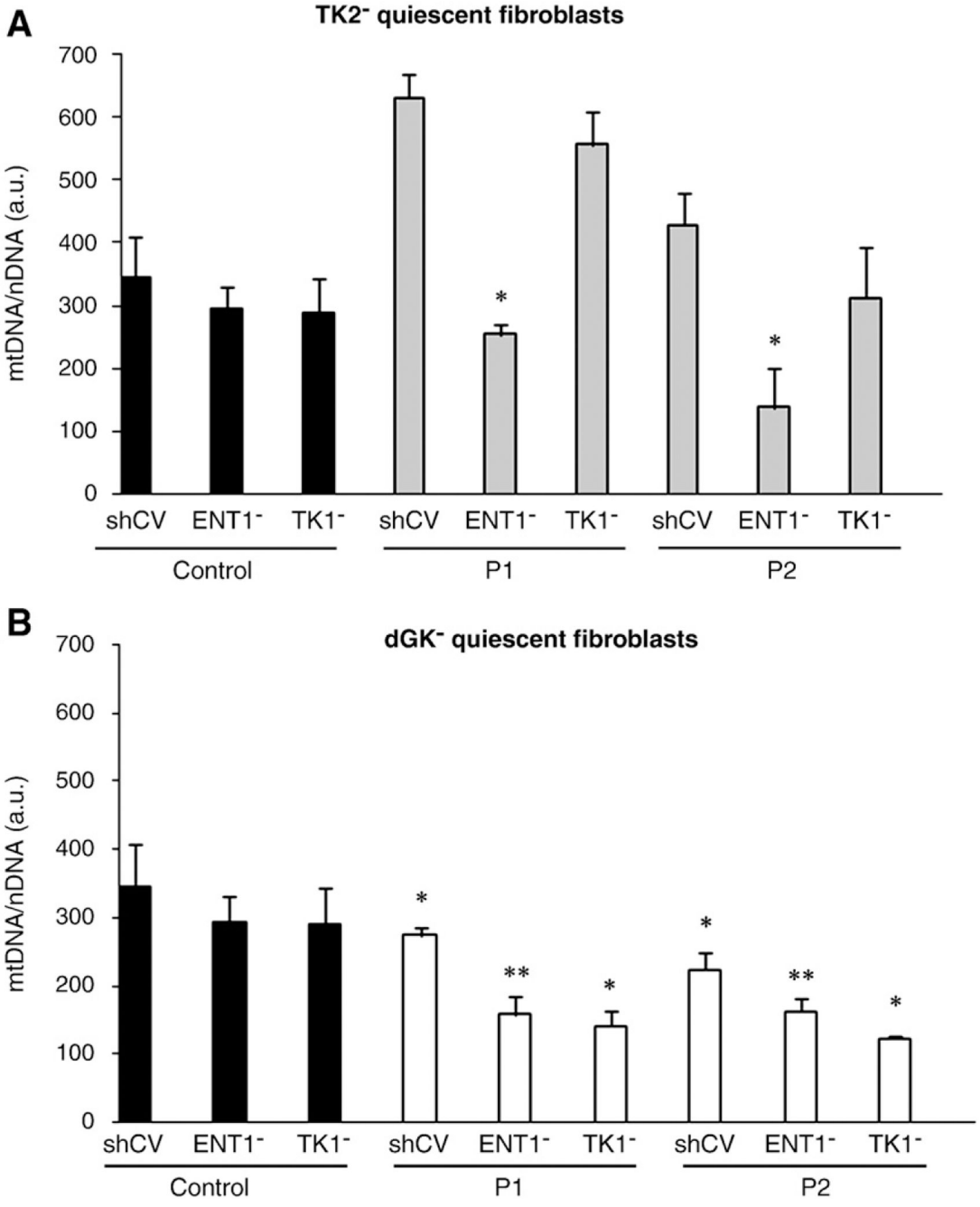

Fig. 5 –.

Measurement of mtDNA depletion in control, TK2, and dGK-deficient fibroblasts lacking hENT1 or TK1 mRNA. Control and TK2 (A), and dGK (B) deficient fibroblasts were infected with lentiviral particles containing antisense small hairpins (shRNA) to down-regulate mRNA expression of hENT1 or TK1, and containing an irrelevant sequence used as an internal control (shCV). After infection, cells were shifted to 0.1% dFBS during 10 days to obtain quiescent cells. The mRNA interfered is indicated below each bar. (*) denotes $p<0.05$, (**) denotes $p<0.001$.

Table 1 –

mRNA expression of genes involved in the maintenance of mtDNA in TK2 and dGK-deficient fibroblasts.

mRNAs	$TK2^{-}$		dGK^{-}	
	P1	P2	P1	P2
Pol γ	0.85±0.02	0.96±0.10	0.35±0.05 *	0.45±0.04 *
TP	1.25±0.12	1.06±0.08	0.11±0.02 **	0.33±0.03 *
TS	0.07±0.00 *	0.44±0.03 *	1.34±0.16	0.73±0.02
TFAM	0.80±0.02	0.69±0.02	0.34±0.02 *	0.38±0.04 *
dGK	1.05±0.02	0.93±0.04	1.39±0.05	1.39±0.01
dCK	0.92±0.02	0.08±0.01	1.13±0.02	1.37±0.03
dNT2	1.01±0.01	0.91±0.02	0.76±0.04	0.92±0.02
nm23-H4	1.34±0.03	1.13±0.02	1.15±0.02	0.97±0.05
nm23-H6	0.96±0.03	0.90±0.04	1.12±0.01	1.22±0.02
ANT1	0.71±0.02	0.81±0.01	0.79±0.01	1.05±0.04
TFB1 M	1.04±0.04	0.96±0.04	0.70±0.03	0.71±0.03
TFB2 M	0.28±0.04	0.30±0.05	0.49±0.02	0.45±0.02

Results are expressed as relative quantification (RQ)±standard deviation (SD).

Control value=1.

*=$p<0.05$.

**=$p<0.001.2$.

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