Conditional knockdown of hMRS2 results in loss of mitochondrial Mg^{2+} uptake and cell death

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Abstract

The human gene *MRS2L* encodes a mitochondrial protein distantly related to CorA Mg^{2+} transport proteins. Constitutive shRNA-mediated knockdown of hMRS2 in human HEK-293 cell line was found here to cause death. To further study its role in Mg^{2+} transport, we have established stable cell lines with conditionally expressing shRNAs directed against *hMRS2L*. The cells expressing shRNA for several generations exhibited lower steady-state levels of free mitochondrial Mg^{2+} ([Mg^{2+}]$_m$) and reduced capacity of mitochondrial Mg^{2+} uptake than control cells. Long-term expression of shRNAs resulted in loss of mitochondrial respiratory complex I, decreased mitochondrial membrane potential and cell death. We conclude that hMrs2 is the major transport protein for Mg^{2+} uptake into mitochondria and that expression of hMrs2 is essential for the maintenance of respiratory complex I and cell viability.

Introduction

Mg^{2+} uptake into the mitochondrial matrix occurs *via* electrophoretic uniport, driven by the (inside negative) membrane potential $\Delta\psi_m$. Intramitochondrial Mg^{2+} concentrations ([Mg^{2+}]$_m$) are kept close to those in the cytoplasm, and thus far below electrochemical equilibrium. This is indicating that Mg^{2+} influx into the organelle is tightly controlled [1, 2]. Earlier we have identified a nuclear gene of the yeast *Saccharomyces cerevisiae,* named *yMRS2,* as encoding a mitochondrial inner membrane protein mediating Mg^{2+} influx into this organelle. Activity of this protein is essential for mitochondrial functions, but yeast cells lacking yMrs2 are viable under fermentative growth conditions [3–5]. This protein is distantly related to the bacterial CorA Mg^{2+} channel protein [6]. Most recently we have shown that yeast Mrs2 forms a high-capacity Mg^{2+}-selective ion channel [6, 7].

The human genome encodes a single expressed homologue (*MRS2L*) of the yeast protein that has been shown to be an integral protein of the mitochondrial membrane. Low levels of the transcript were detected in various tissues. Expression of the human Mrs2 protein (hMrs2) in yeast *mrs2* knockout cells can partially suppress

their mitochondrial dysfunction. Accordingly, yMrs2 and hMrs2 appear to be functional homologues [8].

In order to characterize the role of hMrs2 in mitochondria, we constitutively expressed shRNA directed against *hMRS2* mRNA in HEK-293 cells. Because cells did not survive constitutive knockdown of hMRS2 we utilized conditional expression of shRNA [9]. We obtained clones of a HEK-293 cell line with stably integrated constructs expressing shRNAs directed against human Mrs2 mRNA under the control of doxycycline-regulated promoter. Studies on these conditional knockdown clones revealed that Mrs2 constitutes the major Mg^{2+} uptake system in human mitochondria. Upon Mrs2 ablation, the mitochondria lack complex I of the respiratory chain and cells die.

Materials and methods

Cell culture

Human embryonic kidney cells (HEK-293) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 3.7 g/l NaHCO$_3$ and 110 mg/l pyruvate, supplemented with 8% foetal bovine serum (FBS, GibcoBRL). Additionally, DMEM (4.5 g/l glucose, 3.7 g/l NaHCO$_3$, 8% FBS) without pyruvate and uridine and DMEM (4.5 g/l glucose, 3.7 g/l NaHCO$_3$,

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8% FBS) containing 110 mg/l pyruvate (Invitrogen) and 4 μg/ml uridine (Sigma) were used as mentioned in the text. Cultures were kept at 37°C in 5% CO_2 atmosphere.

Transfection was performed using the SuperFect transfection reagent (Qiagen GmbH, Hilden, Germany). Stably transfected cells were selected in media supplemented with either 200 μg/ml neomycin (Invitrogen Ltd., Paisley, UK) or 50 μg/ml hygromycin (Invitrogen) or 50 μg/ml zeocin (Invitrogen) or 2 μg/ml blasticidin (Invitrogen). The expression of shRNAs was initiated by addition of doxycycline (Sigma, Sigma-Aldrich, St Louis, MO, USA) at the concentration of 1 μg/ml.

Constructs

Synthetic DNA template sequences for shRNAs expression were as follows (complementary sequences underlined):

MRS2-E: **AGATCT**CCACCATGGAATGCCTGCGCTTCAA GAGAGCGCAGGCATTCCATGGTG TTTTTGGAA**AAGCTT** and

MRS2-Q: **AGATCT**CAGCTCGTGAGCTTAGGGTGTTCAA GAGACACCCTAAGCTCACGAGCT TTTTTGGAA**AAGCTT**.

The MRS2-E and MRS2-Q templates were generated by annealing of the sense and antisense oligonucleotides and filling-in by Klenow fragment. The obtained DNA fragments were digested by *Bgl*I and *Hin*dIII and ligated either into pSuper or pTer vectors (both kindly provide by Reuven Agami) [9, 10].

Constructs for tet-inducible shRNA expression were generated by ligation of the *Hin*dIII-*Pst*I fragment harbouring expression cassettes for shRNA-E or -Q from pTer vector into *Hin*dIII-*Pst*I sites of pIND vector with hygromycin selection marker (Invitrogen) yielding ptetHsh-E and ptetHsh-Q. Constructs ptetHsh-E and ptetHsh-Q and the empty parental vector (pIND) were stably transfected into a HEK-293 cell line expressing the tet-repressor (tetR/Neo, derived from pcDNA3.1, Invitrogen, [11]) kindly provided by D. Takai. The resulting clones are referred to as MPL-E1, MPL-Q1 and MPL-O, respectively.

The hMrs2-HA construct in the pcDNA3 vector has been described previously [8]. The hMrs2-HA insert of this vector was cloned into the *Hin*dIII/*Eco*RI sites of the expression vector pIND with the neomycin selection marker. HEK-293 clones MPL-E1 and MPL-Q1, stably transfected with the vectors pVgRXR and pIND-hMrs2-HA have been named MPL-E1/Mrs2-HA and MPL-Q1/Mrs2-HA.

Isolation of mitochondria and Mg²⁺ measurements

Cells were harvested by centrifugation and resuspended in cold isolation buffer (IB) (210 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH pH 7.4 and 1 mM DTT) with 0.25% BSA (fraction V) and 2 mM EGTA/KOH pH 7.4. After digitonin treatment (1 mg/ml) the cells were washed and then broken in a Dounce-homogeniser on ice. Cell debris was removed by repeated low speed centrifugation (300 g for 3 min.). Mitochondria were collected by centrifugation (10.000 g for 20 min.), washed twice with IB buffer with EGTA, recollected again by centrifugation and finally resuspended in IB buffer with EGTA, according to [12].

Measurements of [Mg²⁺] by spectrofluorometry

The determination of free ionised Mg^{2+} concentrations ($[Mg^{2+}]$) in isolated mitochondria by use of mag-fura-2 was done essentially as describe in detail by [5] and in the legend to Supplementary Figure 3. Continuous recordings of fluorescent intensities at excitation 340 and 380 nm (Mg^{2+}-bound and -free dye, respectively) were transformed into 340/380 wavelength ratios by use of the computer program FL WinLab (Perkin-Elmer), and $[Mg^{2+}]_i$ values were calculated from the 340/380 nm ratio according to the formula of Grynkiewicz *et al.* [13], with a dissociation constant 1.53 for the mag-fura 2 –Mg^{2+} complex.

Membrane potential measurements

Cells were loaded with fluorescence dye JC-1 (Molecular Probes) by incubating them with this membrane potential-sensitive dye (final concentration 0.5 μg/ml) in DMEM with 8% FBS for 30 min. at 37°C and 5% CO_2. After washing the fresh DMEM with 8% FBS was added to the cells. Zeiss Axioplan2 microscope was used with appropriate filter sets (emitter/splitter/exciter F41-001, F41-007, F41-020, AF Analysentechnik, Germany): FITC (excitation/emission 460–500 nm/520–570 nm) for JC-1 monomer monitoring (excitation/emission 485 nm/530 nm) and CY3 (excitation/emission 525–565 nm/570–645 nm) for JC-1 aggregate monitoring (excitation/emission ~535 nm/570 nm). Images were captured using a digital charge-coupled device camera (Spot2, Diagnostic Instruments, Inc.).

Gel electrophoresis and immunoblotting

Mitochondria were isolated from cells of clones MPL-O, MPL-E1 and MPL-Q1 grown in the presence of doxycycline for 15 days. Two hundred μg of mitochondria were extracted by addition of extraction buffer (750 mM aminocaproic acid, 50 mM Bistris/HCl pH 7.0) and laurylmaltoside to a final concentration of 1%. After incubation on ice for 30 min., the samples were centrifuged at 100,000 g for 30 min. and the supernatant was supplemented with $^1/_4$ vol. of sample buffer (500 mM aminocaproic acid, 5% Serva blue G). Twenty-five μl of the solubilized protein solution was analysed by blue native (BN) electrophoresis on a 5–18% linear polyacrylamide gradient according to [14]. The gel was blotted onto a nitrocellulose membrane, which was then either stained with Coomassi Blue reagent or analysed by immunoblotting.

To observe shRNA-dependent expression of Mrs2-HA, clones MPL-E1/Mrs2-HA and MPL-Q1/Mrs2-HA were grown first for 36 hrs either in medium with or without doxycycline and then for 24 hrs in the presence of ponasterone, inducing Mrs2-HA expression. Mitochondrial fractions isolated from these cells were separated by SDS-PAGE and immunoblotted with anti-HA and porin (Calbiochem) antisera.

To monitor effects of knockdown of MRS2 expression on complex I, clones MPL-O, MPL-E1 and MPL-Q1 were grown for 0, 5, 10 or 15 days in presence of doxycycline. Mitochondria were isolated and total mitochondrial proteins were separated by SDS-PAGE and immunoblotted using an antiserum against the 39 kD subunit of complex I.

Results

Constitutively expressed shRNA directed against hMRS2

To find effective shRNAs interfering with hMRS2 expression, we have transfected HEK-293 cells with vectors constitutively
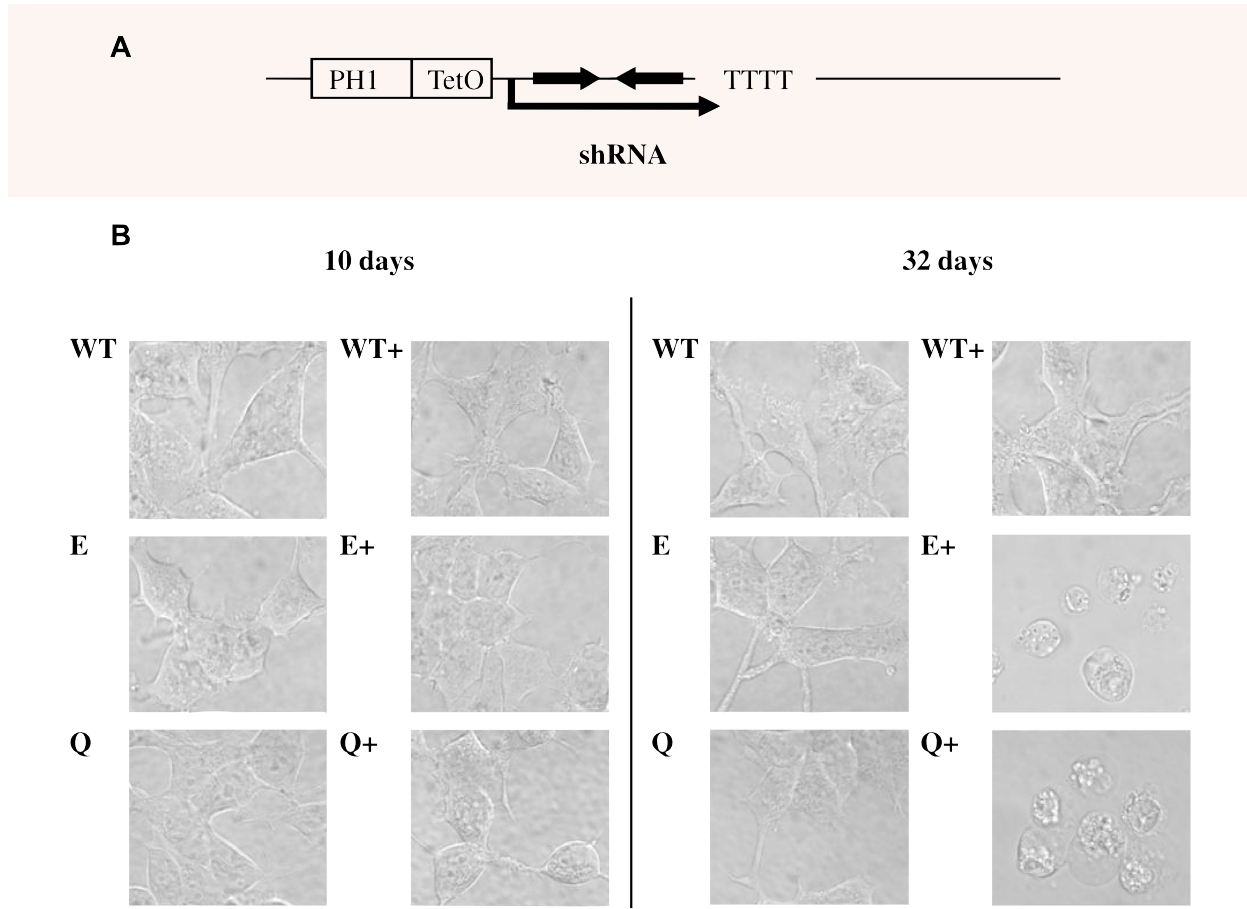
Fig. 1 Conditional knockdown of the Mrs2. (**A**) Overview of ptetHsh constructs for the conditional expression of shRNA-E and shRNA-Q from a tet-regulated H1 promoter (doxycycline binding site tetO downstream of the TATA box). (**B**) Morphology of stably transfected HEK-293 cells carrying vector tetR/Neo and either vector pTer (without shRNA template) or vector ptetHsh-E or vector ptetHsh-Q (marked WT, E and Q, respectively). Cells were grown in the absence of the inducer doxycycline (WT, E and Q) or in the presence of doxycycline (WT+, E+ and Q+) for times indicated. Cell morphology was observed by visible light microscopy.

expressing shRNAs directed against various regions of hMRS2 mRNA (Supplementary Fig. 1A and B). Transient transfection with the pEGFP-Hsh-E and -Q constructs had the strongest effect on the expression of a GFP reporter construct (Supplementary Fig. 1C). In an attempt to obtain stable clones constitutively expressing shRNA-E, we have transfected HEK-293 cells with a construct (pHsh-E) expressing shRNA-E under the control of the H1 promoter (Supplementary Fig. 2A). However, at early stages of post-transfectional selection growth of most clones expressing the shRNA-E was arrested after 14 days. Microscopic inspection of these clones revealed cells with aberrant morphology that were partly detached from the surface of the dish. Vital staining with the lypophilic fluorescence dye JC-1 indicated that most arrested cells were still energized, but many JC-1-loaded daughter cells showed very weak fluorescence of the JC-1 aggregate, indicating

partial loss of the mitochondrial membrane potential $\Delta\psi_m$ (Supplementary Fig. 2B).

Conditional knockdown of hMrs2 expression

To generate conditional hMRS2 knockdown, we have transfected the HEK-293 cells with ptetHsh-E and -Q constructs expressing shRNA under the control of the doxycycline-regulated RNA polymerase III promoter H1 and, in parallel, with a vector expressing the tetracycline repressor (Fig. 1A). Resulting clones MPL-E1 and MPL-Q1 were overall healthy, propagated well and exhibited normal morphology. About 10 days after the activation of shRNA transcription (by doxycycline supplementation), no morphological differences could be detected between the cells growing with or
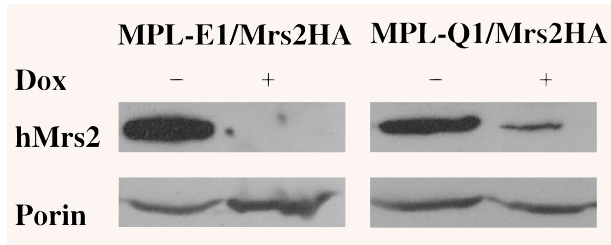
Fig. 2 Down-regulation of Mrs2 protein level by shRNAs. Clones MPL-E1 and MPL-Q1 were transfected with vectors pVgRXR (encoding the ecdysone-VP16 and RXR receptor) and pIND-hMrs2-HA, enabling ponasterone A (Pon, analogue of ecdysone) inducible expression of hMrs2-HA. Stable transfectants were grown for 36 hrs in parallel either with doxycycline (Dox, analogue of tetracycline) or without this inducer and then additionally in the presence of ponasterone for 24 hrs to induce expression of the Mrs-HA protein. Proteins of mitochondrial fractions obtained from these cultures were separated by SDS-PAGE and analysed by immunoblotting using antisera against HA epitope and against porin (outer mitochondrial membrane protein, used as a loading control).

without doxycycline (Fig. 1B). Only when propagated in the presence of doxycycline for 25 days or more clones expressing ptetHsh-E or -Q constructs developed aberrant morphology, rounded up and detached from the surface of the dishes. Control cells transfected with the same vector lacking an shRNA template (MPL-O) stayed healthy during prolonged times of supplementation with doxycycline (Fig. 1B).

Next, we determined the effect of shRNA-E or -Q induction on the expression of hMrs2. Because no hMrs2-specific antibodies are available, we have transfected MPL-E1 and MPL-Q1 clones with pIND-hMrs2-HA/pVgRXR construct and examined by immunoblotting the expression level of hMRS2-HA protein after 2 days' induction of shRNA expression with doxycycline and subsequent overnight induction of Mrs2 transcription with ponasterone in isolated mitochondria. As shown in Figure 2, mitochondria isolated from the cells MPL-E1/hMrs2-HA and MPL-Q1/hMrs2-HA growing in absence of doxycycline had large amounts of Mrs2. In contrast, the clones induced with doxycycline for shRNA expression had much lower levels of Mrs2-HA. Accordingly we conclude that doxycycline-induced expression of shRNA-E or -Q strongly affected the expression of endogenous Mrs2.

Determination of Mg^{2+} uptake rates into isolated mitochondria

Isolated yeast mitochondria have been shown to respond to a rise of external Mg^{2+} ([Mg^{2+}]$_e$), by a rapid increase of intra-mitochondrial free Mg^{2+} concentrations ([Mg^{2+}]$_m$), which can be measured over time by use of the entrapped Mg^{2+}-sensitive dye mag-fura 2 [5]. We have adapted this ratiometric method of [Mg^{2+}]$_m$ determination to HEK-293 mitochondria (cf. METHODS and Supplementary Fig. 3).

Figure 3 presents continuous recordings of [Mg^{2+}]$_m$ obtained with mitochondria isolated from clones MPL-E1, expressing shRNA-E and mitochondria from control cells MPL-O, all grown for 10 days in the presence of doxycycline. In MPL-O mitochondria [Mg^{2+}]$_m$ was calculated to be about 1 mM at resting conditions and to rapidly rise to 2 mM with [Mg^{2+}]$_e$ of 1 mM (Fig. 3). A further increase of [Mg^{2+}]$_e$ to 5 mM led to a minor increase in [Mg^{2+}]$_m$ only (not shown). The response to raising [Mg^{2+}]$_e$ above 1 mM was somewhat variable from experiment to experiment, but generally much less pronounced than the increase observed with [Mg^{2+}]$_e$ of 1 mM. This indicated that the mitochondrial Mg^{2+} uptake system was already saturated at about 2 mM [Mg^{2+}]$_m$. Plateau levels remained constant over extended periods of time, confirming that neither mag-fura 2 leaked in nor magnesium leaked out from the mitochondria. The rapid increase in [Mg^{2+}]$_m$ within a few seconds was comparable to that observed with yeast mitochondria and reflected a high capacity influx driven by the (inside negative) membrane potential $\Delta\psi_m$ of mitochondria.

Mitochondria isolated from shRNA expressing cell lines MPL-E1 exhibited consistently lower [Mg^{2+}]$_m$ values (by 53 ± 17% on average of seven experiments) than in control cells at resting conditions (Fig. 3). Measurements of total cellular Mg^{2+} revealed a small, but statistically not significant decrease of its concentration in clones expressing shRNA-E and shRNA-Q for 10 days (Supplementary Fig. 4). Upon a raise of [Mg^{2+}]$_e$ to 1 mM initial rates of increase in [Mg^{2+}]$_m$ (Δ[Mg^{2+}]$_m$ based on the slope of tangents of the curves within the first few seconds were more than fivefold smaller with MPL-E1 mitochondria than with mitochondria isolated from MPL-O control cells. In these 'mutant' mitochondria the slow increase in [Mg^{2+}]$_m$ lasted for several minutes, but plateau levels finally reached were significantly below those of control cells (not shown). Similar results were obtained with mitochondria isolated from MPL-Q1 cells expressing shRNA-Q for 10 days (not shown).

Stable clones expressing shRNA (MPL-E1, -Q1) as well as control cells (MPL-O) growing in medium supplemented with doxycycline for 10 days were adherent and healthy without any apparent morphological changes. These cells exhibited similar JC-1 fluorescence as cells lacking shRNA expression (Supplementary Fig. 5). Quantitative determination of JC-1 fluorescence signals obtained from isolated mitochondria of the mutant and control cells also revealed similar $\Delta\psi_m$ values (not shown). Thus, a reduction in Mg^{2+} uptake due to weak driving forces could be excluded. Accordingly, shRNA mediated reduction in expression of hMrs2 was regarded as resulting in strongly reduced rates of Mg^{2+} uptake into mitochondria of clones MPL-E1 and –Q1 after 10 days of shRNA induction.

Effects on membrane potential and mitochondrial membrane complexes

Blue native gel electrophoresis was applied to observe effects of down-regulation of Mrs2 expression on mitochondrial membrane

Fig. 3 Determination of free Mg^{2+} concentrations $[Mg^{2+}]_m$ in isolated mitochondria dependent on $[Mg^{2+}]_e$. Mitochondria isolated from clones MPL-O (with empty vector; red curve) and MPL-E1 (expressing shRNA-E; blue curve) after 10 days of doxycycline treatment were loaded with the Mg^{2+}-sensitive dye mag-fura 2. Changes in free Mg^{2+} concentrations $[Mg^{2+}]_m$ over time were determined upon raising extramitochondrial Mg^{2+} concentrations $[Mg^{2+}]_e$ from nominally 0 mM to 1 mM. (For details of methods cf. legend to Supplementary Fig. 3 and [5]).
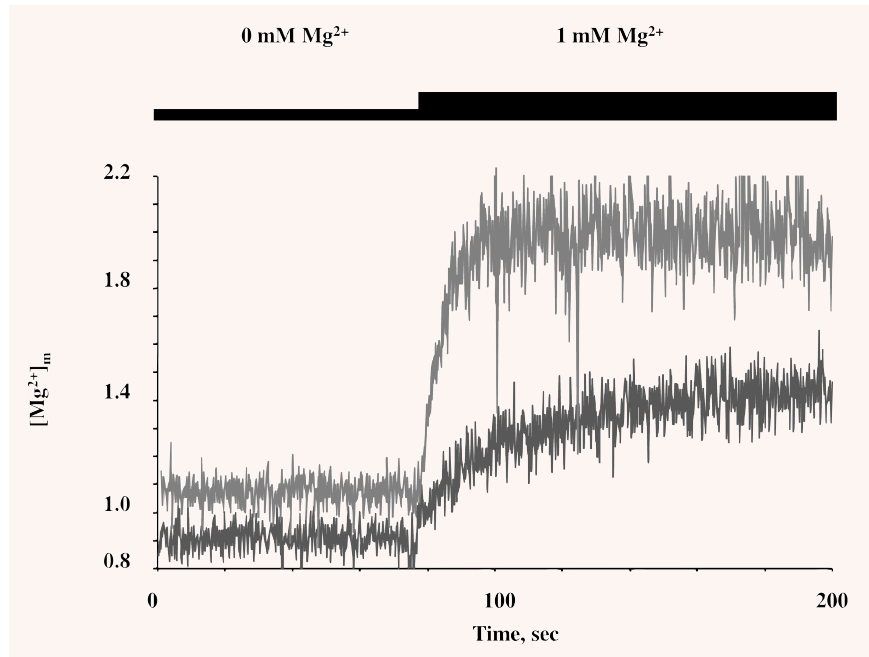
complexes (Fig. 4). Compared to cells not induced for shRNA expression the lack of complex I (NADH:ubiquinone oxidoreductase) was apparent after 15 days of shRNA expression while the pattern of other protein complexes appeared to be unchanged. Western blotting of the blue native gels involving antisera against SU1 of complex I and subunit 4 of complex IV clearly documented the absence of the former complex, but no apparent reduction of the latter. Time-course experiments revealed that amounts of the 39 kD subunit of complex I were slightly decreased 10 days after shRNA induction and strongly decreased upon prolonged shRNA expression (Fig. 5 and Supplementary Fig. 6).

Mitochondrial membrane potential $\Delta\psi_m$ was only marginally reduced after 15 days of shRNA expression and more dramatically after 25 days (not shown). Lack of complex I alone is neither reducing $\Delta\psi_m$ dramatically nor causing cell death in media with abundant glucose [15] because $\Delta\psi_m$ can be maintained *via* ATP hydrolysis by ATP synthase in its reverse mode of activity [16].

Cell death, however, is known to occur upon loss of mitochondrial DNA or blockade of the respiratory chain (though not at complex I) except when media with abundant glucose are supplemented with pyruvate and uridine [17, 18]. We have observed the fate of cells depleted of Mrs2 grown in glucose-containing medium without uridine and pyruvate or in the same medium additionally supplemented with uridine and pyruvate. Both clones MPL-E and MPL-Q showed identical onset of cell death after about 25 days of shRNA expression (not shown). Cell death therefore is not likely due to loss of mtDNA or total loss of respiratory chain components in addition to complex I, but to so far unknown effects of Mrs2 depletion.
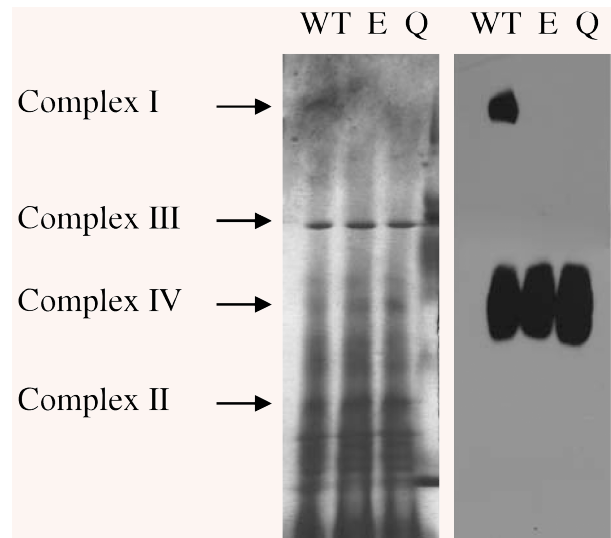
Fig. 4 Effect of Mrs2 ablation on mitochondrial membrane complexes. Clones MPL-O (with an empty vector), MPL-E1 and MPL-Q1 (expressing ptetHshE and ptetHshQ, respectively), marked here as WT, E and Q, were grown for 15 days in the presence of doxycycline. The mitochondria were isolated and proteins were solubilized in laurylmaltoside (1%). Respiratory chain complexes were resolved by BN-PAGE. One part (left) of the gel was stained with Coomassie Blue whereas the other part (right) was transferred onto nitrocellulose membrane and immunodecorated with sera against the 39 kD subunit of the complex I and against subunit IV of the complex IV.
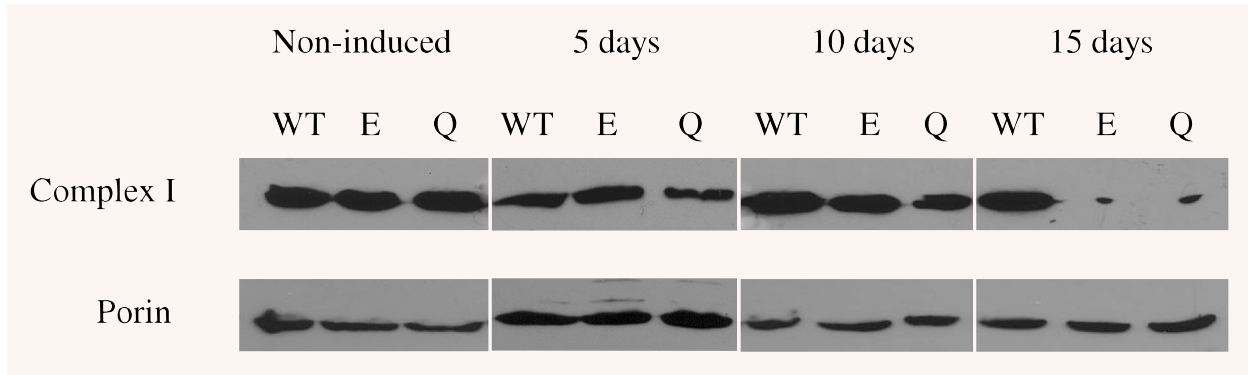
Fig. 5 Down-regulation of Mrs2 expression decreases amounts of 39 kD subunit of complex I. HEK-293 clones MPL-O MPL-E1 or MPL-Q1 (designated as WT, E and Q) were grown for 15 days without doxycycline (non-induced) or for 5, 10 or 15 days in presence of doxycycline. Mitochondria were isolated and total mitochondrial proteins were separated by SDS-PAGE and immunoblotted using an antiserum against the 39 kD subunit of complex I (upper panel) or porin (lower panel).

Discussion

By using the fluorescent Mg^{2+}-sensitive dye mag-fura 2 we have established conditions for ratiometric measurements of free ionized Mg^{2+} concentrations $[Mg^{2+}]_m$ in isolated human mitochondria, which allowed us to detect rapid changes in $[Mg^{2+}]_m$ within short time intervals. Mitochondria of Mrs2 expressing HEK-293 cells responded to the addition of external Mg^{2+} with a twofold increase in $[Mg^{2+}]_m$ which reached plateau levels within a few seconds. We interpret this increase as reflecting rapid influx of Mg^{2+} into the organelles. Maximal $[Mg^{2+}]_m$ levels (of about 2 mM) stayed far below electrochemical equilibrium indicating a tight control of $[Mg^{2+}]_m$.

Earlier attempts to determine changes of $[Mg^{2+}]_m$ dependent on $[Mg^{2+}]_e$ [2], revealed similar large and rapid increases in $[Mg^{2+}]_m$, but only in the presence of the K^+/H^+ ionophore nigericin which converts ΔpH to $\Delta \psi$ in respiring mitochondria. In our assays the membrane potential (as determined by JC-1) as well as $[Mg^{2+}]_m$ increased only slightly upon addition of nigericin, indicating that mitochondria used here were fully energized.

Knockdown of MRS2L in human cell culture clearly resulted in a strong reduction of Mg^{2+} uptake. Initial rates of uptake in response to increases in $[Mg^{2+}]_e$ were about fivefold lower than in the cells not expressing the shRNA. Steady-state $[Mg^{2+}]_m$ values were also lower in mitochondria of knockdown cells, but only by a factor of two. The more modest reduction in steady state levels than in Mg^{2+} influx is explained by the observation that residual Mg^{2+} uptake activity in hMrs2 knockdown mitochondria continues over an extended period of time at a low rate before it finally reaches saturation at a somewhat lower level than in control mitochondria. Knockdown of hMrs2 expression in HEK-293 cells thus had similar effects as *mrs2Δ* knockout mutations in yeast [5].

While the decrease of Mrs2 expression for 10 days caused a considerable loss in Mg^{2+} uptake capacity into mitochondria, cells stayed fully energized, morphologically unchanged and vital. Only when expression of shRNA-E or –Q was induced during 25 days or more, massive cells death could be observed, while doxycycline-treated control cells propagated further and stayed morphologically unaltered for at least 50 days. Mrs2 depletion led to a near complete lack of complex I of the respiratory chain, but has no obvious effects on amounts of other complexes.

In fact, synthesis of mitochondrially made proteins was completely unaffected (our unpublished results). Accordingly, Mrs2 ablation is likely to affect either assembly or maintenance of the large complex I in the inner mitochondrial membrane which is composed of seven mitochondrially encoded and a much larger number of nuclearly encoded proteins. A direct comparison of Mrs2 depletion effects in human and yeast cells is not feasible because yeast lacks a classical complex I of the respiratory chain. Yet yeast *mrs2Δ* cells were found to have a moderate reduction of complex IV of the respiratory chain, resulting either from defects in complex assembly or maintenance in the membrane (our unpublished results).

Mrs2-depleted cells propagated for a considerable period of time in media with abundant glucose after they had essentially lost complex I. Like respiratory deficient human cell lines with mutations in complex I [10, 11] they are assumed to gain ATP by glycolysis and to limit the extent of mitochondrial depolarization by reversal of ATP synthase action. Why Mrs2-depleted cells finally died about 25 days after shRNA induction remained obscure. Loss of mitochondrial DNA (rho^0 mutation) appeared unlikely because addition of pyruvate and uridine to growth media of Mrs2-depleted cells did not prevent their cell death while it is known to prevent cell death of rho^0 cells [12]. Accordingly, so far unknown mitochondrial deficiencies may have caused the phenomenon of delayed cell death in Mrs2-depleted human cells.

The drop of $[Mg^{2+}]_m$ upon Mrs2 depletion observed here in human cells or previously in yeast cells [5, 6] is moderate, but has

surprisingly dramatic physiological effects. Yet, similar or even milder reductions in $[Mg^{2+}]_i$, have previously been shown to cause major functional changes. Lowered $[Mg^{2+}]_i$ caused a bacterial mRNA to undergo conformational changes resulting in transcriptional arrest [19]. Mrs2-depleted yeast cells were reported to arrest mitochondrial RNA splicing [3, 4, 20, 21]. The effects of low free Mg^{2+} concentrations on RNA processing and on the assembly or stability of protein complexes may reflect one of the major known roles of magnesium in biology, namely to stabilize structures of macromolecules. It comes as a surprise that apparently some of these structures would be seriously affected by relatively minor changes in $[Mg^{2+}]_i$.

Acknowledgements

Supplementary Material

The following supplementary material is available for this article:

Fig. 1. Effect of the shRNA on the Mrs2 expression. (**A**) Sequences of the shRNA-E and -Q directed to MRS2 mRNA. The shRNA-E and -Q were targeted to regions from –4 to +15 bp and from +917 to +945 bp (5′ end and central part of coding sequence, respectively) of MRS2L (accession number Q9HD23) mRNA sequence. (**B**) Schematic presentation of the construct pEGFP-Hsh-E used for the expression of shRNA-E from Pol III promoter H1 and of EGFP mRNA from Pol II promoter CMV. The latter transcript contained the MRS2-E sequence as a target for mRNA interference by shRNA-E. The EGFP reporter construct pEGFP/Hsh-E for monitoring of shRNA interfering efficiency was generated by ligation of *Hin*dIII-*Bam*HI fragment harbouring the expression cassette from pSuper vector into *Hin*dIII-*Bam*HI sites of pEGFP-C1 vector with neomycin selection marker (Clontech). (**C**) Fluorescence microscopy of HEK-293 cells revealing shRNA-dependent down-regulation of GFP fluorescence. Confluent cell populations were transiently transfected either with the empty pEGFP-C1 vector or with the pEGFP-Hsh-E constructs. Intensities of EGFP fluorescence were observed 36 hrs after transfection. While pEGFP-C1 transfectants showed bright green fluorescence, pEGFP-shRNA transfectants revealed only barely detectable green fluorescence consistent with the notion that shRNA-E interfered with the reporter GFP-mRNA *via* its MRS2 target sequence. Only a faint signal could be observed upon pro-

longed exposure of the cells transfected with the pEGFP-Hsh-E construct. Similar results were obtained with the pEGFP-Hsh-Q construct (not shown).

Fig. 2. Constitutive Mrs2 knockdown. (**A**) Schematic representation of the construct pHsh-E used for constitutive expression of the shRNA under the constitutive Pol III promoter H1. (**B**) Microscopy of the HEK-293 cells constitutively expressing shRNA-E. Hygromycin selection of pHsh-E transfectants for 14 days resulted in colonies with aberrant morphology (B, left panel). When stained with the lypophilic dye JC-1, most transfected cells showed bright JC-1 fluorescence with a FITC filter (monomeric form of the dye; middle panel) and Cy3 filter (JC-1 aggregates, right panel), essentially reflecting loading of cells with the dye and aggregate formation dependent on the mitochondrial membrane potential $\Delta\psi_m$, respectively. Some daughter cells (circled) showed reduced FITC signals and lacked Cy3 signals indicating loss of mitochondrial membrane potential.

Fig. 3. Original records of the Mg^{2+} uptake measurements. HEK-293 cells stably transfected with (empty) NeoR/Tet and pTer plasmids were grown for 10 days in doxycycline-supplemented medium. Mitochondrial fractions were isolated and loaded with the Mg^{2+}-sensitive fluorescent dye mag-fura 2 (furaptra). (**A**) Loading of mitochondria with the 5 µM membrane permeable mag-fura 2-AM (acetoxymethyl ester) and 5% permeation facilitator Pluronic acid F-127 (both purchased from Molecular Probes) was done in IB buffer with EGTA and 0.5 mM ATP at 25°C for 30 min. Mitochondria were collected by centrifugation (10.000 g for 2 min.), washed twice with IB buffer (without EGTA) and recollected again by centrifugation. The measurements were performed at 25°C in the Perkin-Elmer LS-55 spectrofluorometer with fast filter accessory (FFA), which allowed fluorescence to be measured at 20 ms intervals with emission at 509 nm of free mag-fura 2 and Mg^{2+}-bound mag-fura 2 excited at 380 and 340 nm, respectively, with stirring in 3 ml cuvettes containing 2 ml of isolated mitochondria. Initially the mitochondria were kept in nominally Mg^{2+}-free IB buffer (resting conditions). The stepwise increase of the external $[Mg^{2+}]$ to final concentrations of 1 and 5 mM caused increases in the 340 nm and decreases in the 380 nm excitation curves, reflecting changes of the ion concentration $[Mg^{2+}]$ in mitochondria. For the calibration of the fluorescence signals at the end of each experiment 20 mM Mg^{2+} was added together with triton 80 to rupture the mitochondrial membrane. Release of mag-fura 2 to the buffer led to saturation of the dye with Mg^{2+} that resulted in maximum of 340/380 nm ratio (R_{max}). Finally, addition of EDTA sufficient to chelate all divalent metal ions resulted in minimum 340/380 nm wavelength ratio (R_{min}). (**B**) Continuous recordings of fluorescence intensities at 340 and 380 nm were transformed into 340/380 wavelength ratios by use of the computer program FL WinLab (Perkin-Elmer).

Fig. 4. Total magnesium concentration of MPL-O, MPL-E1 and MPL-Q1 clones. Cells of the MPL-O, MPL-E1 and MPL-Q1 clones grown in the presence of doxycycline for 10 days were resuspended in PBS buffer and permeabilized with digitonin (0.1 mg/ml). Magnesium concentration (± S.D.) of the supernatant obtained after centrifugation at 15,000 rpm was measured by atomic absorption spectroscopy.

Fig. 5. Mitochondrial membrane potential of the cells with downregulated Mrs2. Microscopy of the HEK-293 cells loaded with JC-1. Stably transfected clones MPL-O, MPL-E1 or MPL-Q1 were grown for 10 days in medium supplemented with doxycycline before the cells were loaded with JC-1 to determine their mitochondrial membrane potential $\Delta\psi_m$. The filters FITC and CY3 were used to monitor the monomeric and oligomeric ($\Delta\psi_m$-dependent) forms of JC-1, respectively.

Fig. 6. Amounts of complex I at different time-points after shRNA expression. Mitochondria of clone MPL-E1 (designated here as E) non-induced or grown for different times (10, 11, 12, 13, 14 and 15 days) with doxycycline were isolated and total proteins were separated by SDS-PAGE. Immunoblotting with serum against the 39 kD subunit of complex I (upper panel) or porin (lower panel) was used to see effect of shRNA expression on the amount of complex I.

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