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p28, a truncated form of TRα1 regulates mitochondrial physiology

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ABSTRACT

We have previously identified in mitochondria two truncated forms of the T3 nuclear receptor TRα1, with molecular weights of 43kDa (p43) and 28kDa (p28) respectively located in the matrix and in the inner membrane. Previously, we have demonstrated that p43 stimulates mitochondrial transcription and protein synthesis in the presence of T3. Here we report that p28 is targeted into the organelle in a T3-dependent manner and displays an affinity for T3 higher than the nuclear receptor. We tried to generate mice overexpressing p28 using the human α-skeletal actin promoter, however we found an early embryonic lethality that was probably linked to a transient expression of p28 in trophoblast giant cells. This could be partly explained by the observation that overexpression of p28 in human fibroblasts induced alterations of mitochondrial physiology.

1. Introduction

Triiodothyronine (T3) exerts a pleiotropic effect on development and on adult homeostasis. T3 action is mediated by ligand-inducible transcription factors that are members of the steroid/thyroid hormone receptor superfamily. There are two types of thyroid hormone receptor (T3Rs) encoded by TRα and TRβ genes (NR1A1 and NR1A2 according to nuclear hormone receptor nomenclature) [1,2]. Both TR loci are complex and numerous TR proteins are produced by alternative promoter usage, alternative splicing and the use of internal initiation codon. We have previously identified in mitochondria two truncated forms of the nuclear receptor TRα1, with molecular weights of 43kDa (p43) and 28kDa (p28) respectively located in the matrix and in the inner membrane of mitochondria [3,4].

p43 is a ubiquitously expressed mitochondrial T3 receptor which stimulates mitochondrial transcription and protein synthesis in the presence of T3 [5]. In myoblast, p43 overexpression stimulates mitochondrial activity and potentiates their terminal differentiation [6-8]. Recently, we have generated mice lacking specifically p43 [9] or mice overexpressing p43 in skeletal muscle [10]. We have shown that p43 affects muscle mass and the meta-bolic and contractile features of myofibers [10–12]. In addition, we also found that p43 depletion induced a major defect in insulin secretion and a loss of glucose-stimulated insulin secretion [9], and that these mice became glucose intolerant and insulin-resistant during aging [13]. These data demonstrate clearly the physiological importance of the mitochondrial T3 receptor p43.

Apart for its localization in the mitochondrial inner membrane [3,4] we know little about the other mitochondrial T3 receptor p28. Here we report that this protein is targeted into the organelle in a T3-dependent manner. In addition, binding experiments indicate that p28 displays an affinity for triiodothyronine higher than p43 and the T3 nuclear receptor. We tried to generate mice over-expressing p28 in skeletal muscle using the human α-skeletal actin promoter. However we found an early embryonic lethality linked to a transient expression of p28 in the developing placenta. This could be partly explained by the observation that overexpression of p28 in human fibroblasts induced alterations of mitochondrial physiology.

2. Materials and methods

2.1. Mitochondrial import

Import experiments were performed as previously described [14] using highly purified isolated rat liver mitochondria. Mitochondria were incubated for 45 min in the presence of 5% rabbit reticulocyte lysate containing [^{35}S]-methionine-p28 or [^{35}S]-

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methionine-RXRα (Promega Kit). After import experiments, mitochondria were treated with proteinase K to avoid contamination by non-imported proteins as previously described [5]. 10% of the amount of reticulocyte lysate added to mitochondria for import experiments was loaded in the control lane.

2.2. T3 binding assays

p28 affinity for T3 (Ka) was measured in saturation experiments using [^{125}I]-T3 (3.3 mCi/µg; NEN Life Science Products) according to Daadi et al. [15]. Non-specific binding was assessed in simultaneous assays in which 1 µM of cold T3 was added. Bound and free T3 were separated using Sephadex G-50 column.

2.3. Transgene construct

The mouse p28 coding sequence was amplified using the following primers: 5′mp28, AGG ATC CAG GAG GAG ATG ATT CGC TCA CT; 3′mcea1250, AAG ATC TGC CGC CTG AGG CTT TAG A, and cloned in a pGEM-T vector. The 1.0 kb EcoRI fragment from pGEM-mp28 was inserted in the pGS-HSA plasmid (kindly provided by Melki) [16] containing the 2.2 kb fragment of the human α-skeletal actin promoter (HSA) [17,18], a beta globin intron and SV40 polyadenylation site. The NotI fragment of the pGS-HSA-mp28 plasmid was inserted in pBS3isol plasmid containing three copies the 5′HS4 fragment from the chicken β-Globin gene locus (kindly provided by Houdebine) [19].

2.4. Ethics statement

All animal experiments were performed according to European directives (86/609/CEE). Our institution guidelines for the care and use of laboratory animals were respected. Our animal facility is approved by the Departmental Veterinary Services (No. C34-172-10) and our Ministry of Research (No. 4962). In addition the corresponding author also has an authorization to experiment on living vertebrates certified by the departmental veterinary services (No. 34356). The experiments described in this manuscript did not need a special authorization from our ethics committee other than those listed above.

2.5. Generation of founders and screening

The transgene was excised from pBSisol/HSA-mp28/2isol on a KpnI fragment. DNA was micro-injected into fertilized oocytes of F1 mice (C57BL/6JxCBA). Screening of founders mice and their offspring for stable transmission through the germline was done by PCR and/or Southern-Blotting. DNA was extracted from tail biopsy in lysis buffer (100 mM Tris–HCl pH7.5, 1 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg proteinase K) overnight at 56 °C followed by phenol extraction, isopropanol precipitation and resuspension in H2O. A 586 bp fragment corresponding to the endogenous c-erb A α locus was amplified using the following primers: 5′mp28, AGG ATC CAG GAG GAG ATG ATT CGC TCA CT; 3′intron 6, CTT GGT GTT GGG TAA CTT AGT GCA. A 344 bp fragment corresponding to the transgene was amplified using the following primers: 5′mp28, AGG ATC CAG GAG GAG ATG ATT CGC TCA CT; 3′ceaE7, CTC GGA GAA CAT GGG CAG TTT T. The PCR was performed in Promega reaction buffer containing 400 ng of genomic DNA, 1.5 mM MgCl2, 0.3 µM of each primer, 0.3 mM of each dNTP and 1.25u of TAQ polymerase in a final volume of 25 µl. Thirty-five cycles were performed with an annealing temperature of 56 °C. For Southern-Blot analysis, 10 µg of total genomic DNA was digested using EcoRI restriction enzyme and transferred to a Nytran supercharge membrane (Schleicher & Schuell) following standard procedures.

2.6. Histological analysis

After euthanasia of mothers by cervical dislocation, mouse embryos were collected at E6.5, embedded with OCT matrix, and immediately frozen in isopentane cooled in liquid nitrogen. Ten µm thick serial sections were obtained and processed for immuno-histochemical staining with RHTII antibody raised against thyroid hormone receptor. Briefly, the embryo sections were incubated with the antibody for 1 h at 37 °C. After washing with phosphate-buffered saline, the second antibody, rabbit anti-mouse IgG labeled with rhodamine (Fluoprobes) diluted 1:50 v/v in phosphate-buffered saline, was applied for 30 min at 37 °C. After further washing, the sections were fixed with moviol. Nuclei were stained with Hoechst 33258 (1 µg/ml).

2.7. Cell culture

Human dermal fibroblasts were grown in DMEM supplemented with gentamicin (100 IU/ml) and FCS (10%) at 37 °C and 5% CO$_2$ as previously described [20]. Human fibroblasts constitutively expressing p28 were obtained by stable transfection of the pIRV-p28 expression vector. Control fibroblasts were obtained by stable transfection of the pIRV "empty" vector. Ten micrograms of each plasmid carrying G418 resistance were transfected using the calcium phosphate procedure 24 h after plating. The medium was changed 24 h later after PBS washes, and amplification was done after 10 days in the presence of G418.

2.8. Measurement of cytochrome oxidase activity

Cells plated in coated dishes were harvested in 1 ml PBS and then centrifuged for 5 min at 12000×g. For enzymatic activity determination, the pellet was resuspended in 100 µL lysis buffer [10 mmol/L Tris (pH 7.8)] and lysed by three cycles of freezing/defreezing. Total proteins were measured on an aliquot using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Cytochrome oxidase was measured as specific activity [21].

2.9. Cytoimmunofluorescence studies

Mitochondrial morphology was assessed by cytoimmunofluorescence. After methanol fixation and appropriate washings, cells were stained with a mouse anti-human mitochondria monoclonal antibody (MAB1273, Chemicon International, Temecula, CA) and then incubated with a rhodamine-conjugated antibody raised against mouse immunoglobulins.

2.10. Apoptosis

Quantification of cell apoptosis was measured using Annexin V FITC (BD Bioscience), which utilizes Annexin V-PE to detect the phosphatidylserine on the external membrane of apoptotic cells. Briefly, 100 µl of 10^6 cells was suspended in a mixture of 100 µl Annexin V-PE. After incubation at room temperature for 15 min, samples were analyzed by flow cytometry. Results are expressed as percentage of apoptotic cells.

3. Results

3.1. p28 displays a binding affinity for T3 higher than p43 and the nuclear receptor TRα1

We have previously shown that p43 displays a binding affinity for T3 similar to that of the nuclear receptor TRα1 [5]. In order to define the p28 binding affinity for T3 we performed saturation
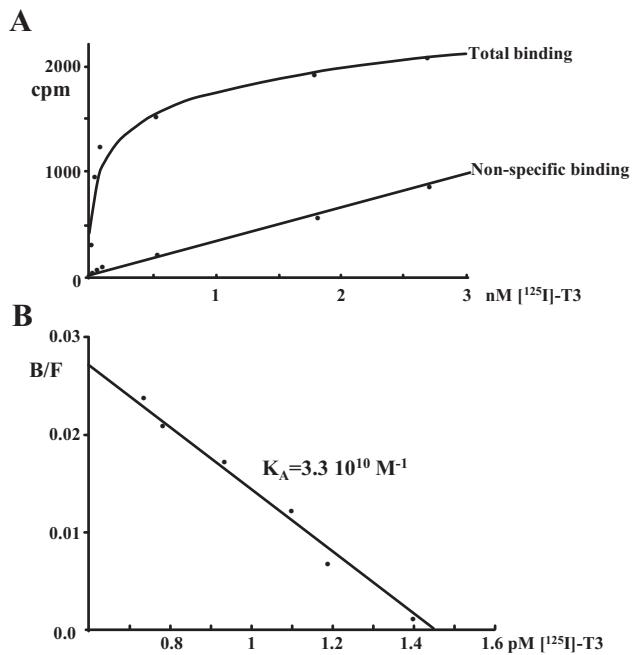
A

B

Fig. 1. Binding affinity of p28 for triiodothyronine. (A) Saturation experiments were performed with p28 synthesized in rabbit reticulocyte lysate and labeled with [^{125}I]-T3. (B) The affinity constant (Ka) was estimated by using a Scatchard linearization of saturation experiments. Data are the mean ± S.E.M. of three separate experiments.

experiments using [^{125}I]-T3. After Scatchard Analysis, we found that p28 displayed a strong affinity (Ka = 3.3×10^{10} M^{-1}) for the hormone (Fig. 1), higher than p43 and TRα1 (respectively Ka = 2×10^9 and 3×10^9 M^{-1}) [5,22].

3.2. p28 is imported into mitochondria in a T3 dependent manner

To understand how p28 is addressed to mitochondria, we studied the import of [^{35}S]-p28 in isolated rat liver mitochondria as previously described by our team [5,23,24]. In these experiments, RXRα which is cleaved during import was used as control [23]. At the end of the import experiment, the reaction mixture was treated with proteinase K to rule out contamination by non-imported proteins. In these conditions, we found that p28 is protected against proteolytic activity inside the organelles only in the presence of T3 (Fig. 2A, lane 3) as RXRα (Fig. 2C, lane 3). The observation that, following import, p28 and RXRα sensitivity to proteinase K is restored after solubilization of mitochondrial membranes by a detergent treatment (Fig. 2B, lane 3 and Fig. 3C, lane 4), demonstrated that protection of p28 and RXRα proteins to the peptidase enzyme is provided by mitochondrial membranes. Therefore, these data establish that p28 is translocated into mitochondria in a T3 dependent manner.

3.3. p28 overexpression induced early embryonic lethality

In order to assess the importance of p28 in muscle development and of mitochondrial activity, we planed to generate mice overexpressing this mitochondrial T3 binding protein under control of the 2.2-kb human α-skeletal actin promoter (HSA) flanked by chicken β-globin 5′HS4 insulator (Fig. 3A) as was previously done with the p43 mitochondrial T3 receptor [10]. The transgene construct was micro-injected into fertilized oocytes of F1 mice (C57BL/6JxCBA) and viable pups were screened by Southern-Blot and PCR (Fig. 3B and C). 26 mosaic p28-Tg founders mice were obtained (15 males and 11 females).

Mosaic p28-Tg founders mice were crossed to normal C57BL/6 mice to generate transgenic animals. Unfortunately, no p28-Tg pups were identified in 153 litters born comprising 1173 pups,
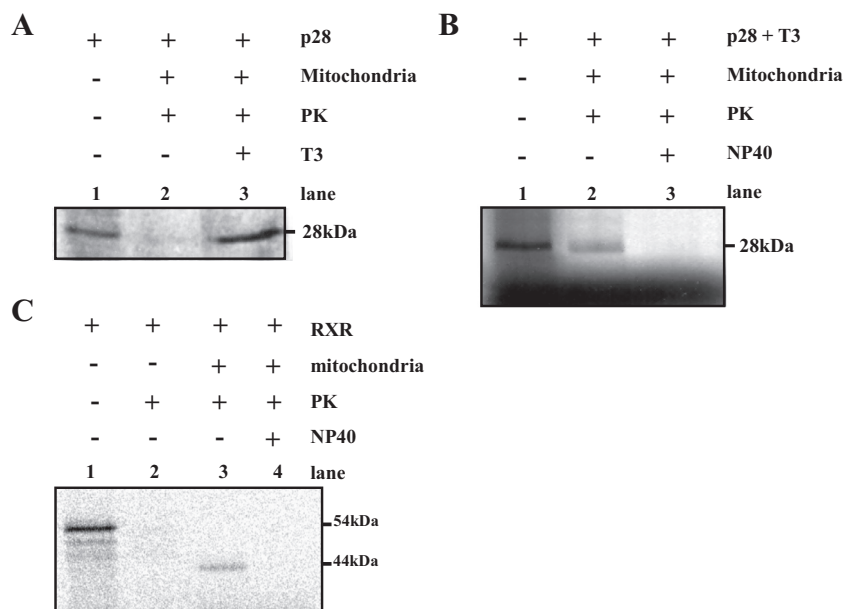

Fig. 2. p28 is imported into mitochondria in a T3-dependent manner. Import experiments were performed in isolated mitochondria using [^{35}S]-p28 or [^{35}S]-RXRα synthesized in rabbit reticulocyte lysate. At the end of the import experiment, the reaction mixture was treated with proteinase K to rule out contamination by non-imported proteins. (A) Lane 1: p28 alone; lanes 2: p28 is not imported into mitochondria; lane 3: p28 in presence of T3 is internalized in mitochondria as shown by the acquired protection against proteinase K added at the end of the experiment. (B) Lane 1: p28 alone; lanes 2: p28 in presence of T3 is internalized in mitochondria as shown by the acquired protection against proteinase K added at the end of the experiment; lane 3: at the end of the experiment, protection of p28 against proteinase K is abolished by solubilization of mitochondrial membranes by tris-NP40. (C) Lane 1: RXRα alone; Lane2: RXRα is sensitive to proteinase K; Lane 3: RXRα is internalized in mitochondria as shown by the acquired protection against proteinase K added at the end of the experiment; Lane 4: at the end of the experiment, protection of RXRα against proteinase K is abolished by solubilization of mitochondrial membranes by tris-NP40. When indicated, 10^{-9} M T3 was added.
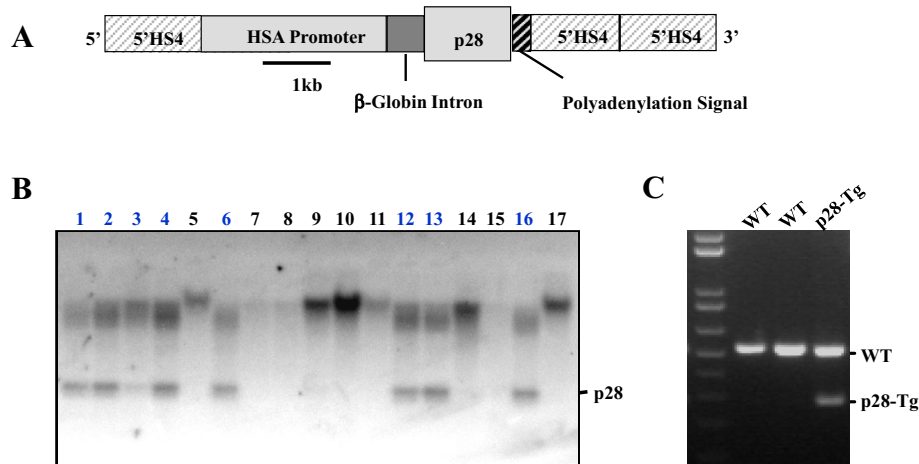
Fig. 3. Generation of transgenic founders mice. (A) Schematic representation of the construct used for microinjection of [C57BL/6 x CBA] F1 fertilized oocytes. HSA: human α-skeletal actin; 5′HS4: chicken β-globin insulator. (B) DNA were isolated from tail from several transgenic founders mice and subjected to hybridization analysis with probes for p28. 20 μg of total DNA was analyzed. (C) PCR analysis of wild-type mice and transgenic founders.

Table 1
Distribution of viable pups/litter resulting from matings of p28-Tg founders mice.

Number of viable pups/litter	Number of founders
<6,5	7
6,5–7	3
7–7,5	3
7,5–8	0
8–8,5	5
8,5–9	4
>9 h	4

A

Age	N=	Genotype	
		+/+	Tg
E7-E8.5	55	40	15
E9.5-E12.5	91	91	0

B

Fig. 4. p28 overexpression induced early embryonic lethality. (A) Genotype analysis of embryos with different gestational ages; (B) Picture showing a wild-type and a transgenic embryos at E8.5.

suggesting an embryonic lethality. Consistent with this possibility we found that some founder mice had a smaller number of young per litter ($n < 6.5$) compared to the normal average ($n = 8$) (Table 1). Three males of these founder mice were crossed to normal C57BL/6 mice and embryos were recovered at different stages of gestation and genotyped by PCR. Interestingly, we found transgenic embryos with gestational ages <E8.5 (15/55) (Fig. 4A). However, p28-Tg embryos with gestational ages ⩾E9.5 were not identified (0/91) (Fig. 4A). Examination of embryos of various gestational ages revealed that p28-Tg embryos appeared to develop normally until E8.5, after which development is stopped, followed by degeneration (Fig. 4B).

3.4. p28 overexpression in developing placenta

This early embryonic lethality suggested that the transgene could be expressed in extra-muscular tissue. In agreement with this possibility Clausen et al. [25] reported that α-skeletal actin is transiently expressed in placenta. This observation led us to investigate p28 overexpression in this tissue. To this end, we performed immunofluorescence studies in transgenic and control embryos sections from E8 gestational stage using an antibody raised against thyroid hormone receptor TRα1. This antibody is not specific to p28 and recognizes p43 and TRα1. As expected, we found that placenta from transgenic embryos overexpressed a TRα1 protein. In particular, the protein was expressed in trophoblast giant cells, which are polyploid cells, and presented a punctiform network like mitochondria (Fig. 5). However, the protein was not overexpressed in other embryonic tissues (Data not shown).

3.5. Mitochondrial physiology was affected by p28 overexpression in human fibroblast

To assess the possible influence of p28 on mitochondrial physiology, we stably transfected the protein in human fibroblasts as previously described for p43 [20]. As attested by Western blot, two clones with distinct levels of p28 were obtained (Fig. 6A). Further experiments were conducted using the clone 2 which showed a higher level of p28. Cytoimmunofluorescence studies performed using an antibody raised against mitochondria, revealed a profound alteration of the mitochondrial morphologies in p28 overexpressing cells in comparison to control cells (Fig. 6B). Wild-type fibroblasts were characterized by a mitochondrial network of extended tubules distributed throughout the cytoplasm with
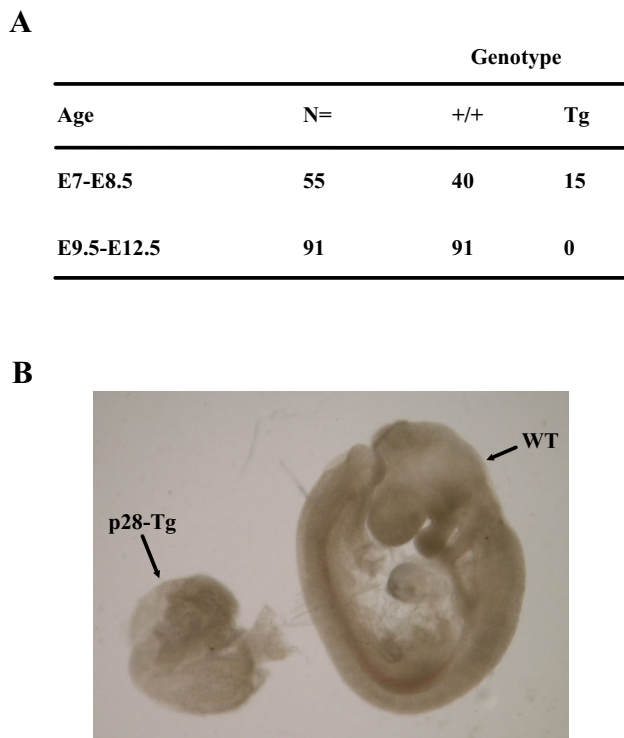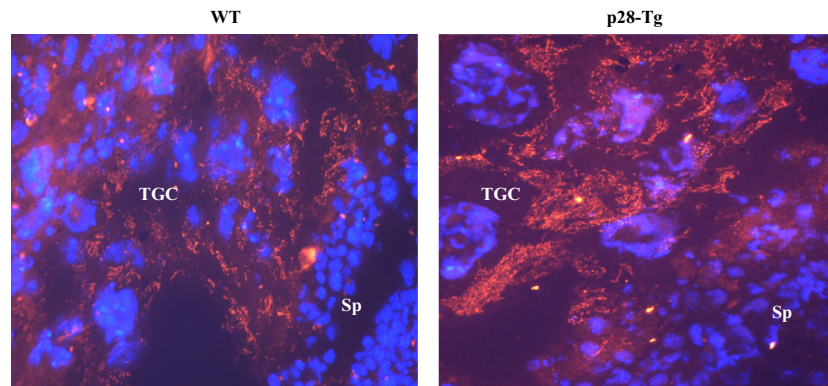
Fig. 5. Overexpression of p28 in developing placenta. Immunofluorescence staining of serial embryo sections showing p28 overexpression (red). Nuclei were stained with Hoechst (blue). The p28 overexpression is mainly restricted to the trophoblast giant cells. TGC: trophoblast giant cell; Sp: spongiotrophoblasts.
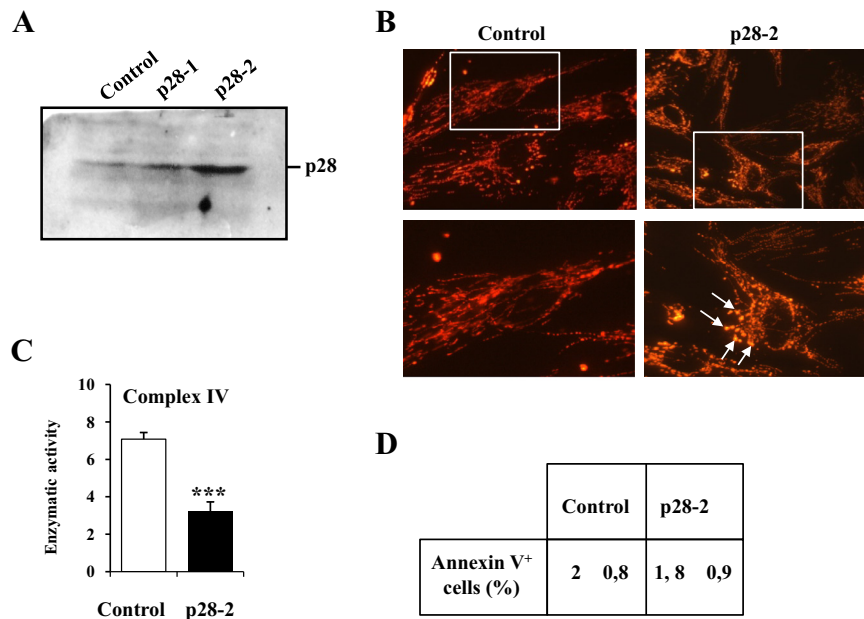

Fig. 6. Overexpression of p28 in human fibroblasts induced alteration of mitochondrial physiology. (A) p28 protein levels in human fibroblasts stably transfected by pIRV (control) or pIRV-p28 expression vectors (p28-1 and p28-2), visualized by western-blot using an antibody raised against TRα. 50 μg of total protein extracts were analyzed. (B) cytoimmunofluorescence experiments performed using an antibody raised against mitochondria in control and p28-2 cells. Arrows indicate big spherical mitochondria. (C) Cytochrome oxidase activity (Complex IV) measured in control and p28-2 cells. Data represent the mean ± S.E.M. of three independent experiments (***$P < 0.001$). (D) Apoptosis of control and p28-2 cells. Cells were harvested and stained with annexin V FITC for flow-cytometric analysis. Data represent the mean ± S.E.M. of three independent experiments.

only a few rounded mitochondria present (Fig. 6B). In contrast, in fibroblasts overexpressing p28, we observed a strong accumulation of rounded mitochondria and only a few significant tubules were detected (Fig. 6B). In addition, in these cells, we found a strong inhibition of cytochrome oxidase activity (complex IV) in fibroblasts overexpressing p28 (−68%, $P < 0.001$) (Fig. 6C). Finally, to determine whether p28 could influence apoptosis, the Annexin V-FITC detection kit was used. We found that the extent of apoptosis was very low and not significantly affected by p28 overexpression (Fig. 6D).

4. Discussion

According to Bigler et al. [26], p28 is synthesized by the use of an internal AUG of the TRα1 messenger. In contrast to p43 and the nuclear receptor TRα1, p28 lacks the DNA binding domain of these receptors. We observed that the binding affinity of p28 for T3 was higher than for p43 and TRα1 [5,22]. In addition, this affinity is identical to that previously found for specific T3 binding sites localized in the inner mitochondrial membranes [27,28]. This suggests that p28 probably corresponds to the specific T3 binding sites characterized by these authors. Moreover, these results also suggest that the amino-terminal deletion occurring in p28 increases the T3-binding activity of the protein.

Import experiments using isolated mitochondria revealed that p28 like p43 is targeted to mitochondria. However, in contrast to p43, p28 is imported into the organelle in a T3 dependent manner. This data and the difference in their localization in the mitochondria suggest that p28 and p43 probably use distinct import pathways. In addition, this T3-dependent localization raises the

question of whether the hormone is important for the function of p28, or if the hormone is only required for the import of the protein in the mitochondrial membrane.

If T3 is often considered as the major active iodothyronine, accumulating evidence suggests that other iodothyronines such as 3,5-diiodothyronine (T2) could have biological relevance. In particular, T2 is able to stimulate mitochondrial activities [29,30] and specific binding sites for T2 have been described in rat liver mitochondria [31]. In addition, these authors have shown that T2 could interact with the cytochrome c complex [30]. The data associated with the mitochondrial inner membrane localization of p28 suggest that p28 could also interact with this thyroid hormones derivative. However, the binding affinity of p28 for T2 remains to be elucidated as well as whether or not T2 has a physiological function.

To directly assess in vivo the role of p28, we tried to generate transgenic mice expressing p28 under the control of the HSA muscle specific promoter [16,17] flanked by chicken β-globin 5′HS4 insulator. Insulators are used to reduce variation between founder transgenic mice and to avoid having mice that fail to transmit the transgene to their progeny [32]. Using the same strategy we have previously generated several transgenic lines with various levels of p43 overexpression (2-fold to 8-fold) [10]. Unfortunately, in this study despite the obtention of 26 chimeric p28-Tg founders mice, the birth of 153 litters and 1153 pups, no viable p28-Tg mice were obtained, suggesting an embryonic lethality. Because the probability that all p28 founders mice only allowed very high levels of p28 expression in progeny was clearly unlikely, we excluded the possibility that the embryonic lethality observed was an artefact linked to very high levels of p28 overexpression.

One possibility was that the very high T3 binding affinity of p28 could result in an early lethality caused by a T3 sequestration by p28. However, we excluded this possibility because if insufficient T3 levels during pregnancy in humans results in intellectual impairments, growth retardation, neonatal hypothyroidism, and an increase of pregnancy loss and infant mortality, hypothyroidism has never been considered as inducing a systematic embryonic lethality as observed in our study.

Examination of embryos revealed interesting data. First, we found numerous transgenic embryos with gestational ages <E8.5. Moreover, at this stage transgenic embryos and control embryos could not be morphologically distinguished before E8.5. At E8.5 development of transgenic embryos was stopped followed by degeneration thus explaining why embryos with gestational ages ⩾E9.5 were not identified.

Localization of p28 overexpression in E7.5 placentas revealed that the protein was highly expressed in trophoblast giant cells. Unfortunately, we cannot show pictures demonstrating the co-localization of p28 in the mitochondria of trophoblast cells because we no longer have the transgenic embryos sections. However, in trophoblast giant cells the protein was in a punctiform network like mitochondria and clearly not localized in the nucleus or at plasma membrane. Trophoblast giant cells are polyploid cells which are the first cells to differentiate from the trophoectoderm, but they may also derive from trophoblasts and spongiotrophoblasts via endoreplication [33], a process often associated with highly metabolically active cells [34]. Trophoblast giant cells invade into the uterine wall, establishing a receptive environment for development of a functional maternal-fetal interface via elaboration of paracrine factors including VEGF, metalloproteinases, hormones and cytokines [35,36]. Thus, viable trophoblast giant cells through their role in the diffusion of nutrients and oxygen between maternal and fetal blood and the production of different growth hormones are essential for embryo implantation and placental development [35]. By consequence, the early embryonic lethality induced by p28 may reflect a giant cells deficiency.

Because of their high metabolic rate, trophoblast giant cells may be particularly vulnerable to perturbations in mitochondrial dynamics and in respiratory chain activity. Our data obtained on fibroblasts showing that p28 overexpression induced a strong decrease of cytochrome c oxidase activity and an alteration of mitochondrial network, suggest that the embryonic lethality could be directly linked to alterations of mitochondrial physiology in trophoblast giant cells.

Very recently, in osteocytes, a TRα1 protein, produced from internal translational start codon (met 150) as p28, was found to be located at the plasma membrane level and activate kinase cascades to regulate cell survival and proliferation [37]. In addition, they found that the palmitoylation of this TRα protein is strictly required for the plasma membrane localization. This report suggests that TRα, as others receptors belonging to the steroid receptor superfamily (Estrogens (ER), androgens (AR), and progesterone receptors (PR)), exerts rapid, non-genomic effects through membrane-bound receptors (For review: [38]) in a palmitoylation dependent fashion. Interestingly, a recent study using a transgenic mouse with a point mutation of the palmitoylation site of ERα (C451A-ERα) to obtain membrane-specific loss of function of ERα, demonstrate that palmitoylation of ERα, which is best known to date, is tissue-specific and regulates membrane versus nuclear actions in vivo [39]. In particular, in this study the authors show that E2 action in the uterus was preserved in C451A-ERα mice suggesting that palmitoylation of proteins belonging to nuclear receptor family does not occur in this tissue. Thus probably because DHHC-7 and -21, two palmitoylacyltransferase involved in the endogenous ER, PR, and AR palmitoylation [40] are not expressed in this tissue. Our data and the study of Kalyanaraman and co-workers [37] suggest that p28 is located at the mitochondrial level or sometimes at the plasma membrane in tissues where palmitoylation event occurs. However, because in our study the overexpressed 28kDa TRα1 protein was clearly not localized at the plasma membrane in the trophoblast giant cells of uterus, the embryonic lethality observed is probably linked to a mitochondrial defect induced by the mitochondrial localization of the protein.

In future studies, it will be of interest to explore potential physiological roles of p28 in vivo. These studies will require generation of mice overexpressing p28 in selected lineages and/or at selected periods of development and the availability of specific knockout mice. The importance of elucidating p28 function in vivo is highlighted by our studies demonstrating the physiological importance of p43, clearly defined as a mitochondrial T3 receptor [9–13,41].

Acknowledgment

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