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Torin1 restores proliferation rate in Charcot-Marie-Tooth disease type 2A cells harbouring MFN2 (mitofusin 2) mutation

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Objective. Mitofusin 2 (MFN2) is a mitochondrial outer membrane protein that serves primarily as a mitochondrial fusion protein but has additional functions including the tethering of mitochondrial-endoplasmic reticulum membranes, movement of mitochondria along axons, and control of the quality of mitochondria. Intriguingly, MFN2 has been referred to play a role in regulating cell proliferation in several cell types such that it acts as a tumour suppressor role in some forms of cancer. Previously, we found that fibroblasts derived from a Charcot-Marie-Tooth disease type 2A (CMT2A) patient with a mutation in the GTPase domain of MFN2 exhibit increased proliferation and decreased autophagy. Methods. Primary fibroblasts from a young patient affected by CMT2A harbouring c.650G > T/p.Cys217Phe mutation in the *MFN2* gene were evaluated versus a healthy control to measure the proliferation rate by growth curves analysis and to assess the phosphorylation of protein kinase B (AKT) at Ser473 in response to different doses of torin1, a selective catalytic ATP-competitive mammalian target of rapamycin complex (mTOR) inhibitor, by immunoblot analysis. Results. Herein, we demonstrated that the mammalian target of rapamycin complex 2 (mTORC2) is highly activated in the CMT2A^{MFN2} fibroblasts to promote cell growth via the AKT(Ser473) phosphorylation-mediated signalling. We report that torin1 restores CMT2A^{MFN2} fibroblasts' growth rate in a dose-dependent manner by decreasing AKT(Ser473) phosphorylation. Conclusions. Overall, our study provides evidence for mTORC2, as a novel molecular target that lies upstream of AKT to restore the cell proliferation rate in CMT2A fibroblasts.

Key words: Charcot-Marie-Tooth type 2A2, mitofusin2, AKT, cell proliferation, mTORC2

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Introduction

Charcot-Marie-Tooth disease type 2A (CMT2A) (OMIM 609260), is an autosomal dominant inherited sensorimotor neuropathy affecting peripheral nerve axons, that has causative mutations in the mitofusin 2 (*MFN2*) gene located in chromosome 1 (chr1:11.998.820). This gene encodes for mitofusin 2 (*MFN2*) protein which is related to dynamin family GTPases. MFN2 protein has pleiotropic cellular roles, which include participation in mitochondrial fusion, mitochondria–endoplasmic reticulum

tethering, mitochondrial trafficking along axons, and mitochondrial quality control [1]. MFN2 is also involved in the regulation of cell survival and for this reason, it has been of interest in the cancer field [2]. Cellular proliferation is closely dependent on the dynamics of mitochondria as it has been shown that high levels of mitochondrial fission are associated with active proliferation and the maintaining of mitochondrial hyper-fused morphology can regulate the cell transition from G1 to the S phase [3,4]. To date, several studies in CMT2A harbouring a mono-allelic mutation in MFN2 with autosomal dominant inheritance have not been conclusive on the molecular mechanisms causing cellular alterations. Efforts have been mainly focused on respiratory chain capacity, oxidative phosphorylation [5,6], mitochondrial membrane potential [6] or mitochondrial DNA (mtDNA) content [5,7], reporting extremely variable results, whereas most of the studies about the role of MFN2 in autophagy and proliferation have been performed in tumour cells. In a previous study, we analyzed both the mitochondrial and cellular phenotypes in CMT2A^{MFN2} fibroblasts harbouring a monoallelic MFN2$^{650G>T/C217F}$ mutation in the GTPase domain [8,9], which has been classified as "likely pathogenic" from the ACMG [10]. We found that CMT2A^{MFN2} fibroblasts presented an increase in the so-called intermediate-fragmented mitochondria; an inefficient capacity in recovering mitochondria morphology upon removal of a stressful insult; the depolarization of the mitochondrial membrane, and impaired respiration due to a significant reduction of respiratory complexes' activities. Hence, we asked whether the presence of damaged mitochondria in CMT2A^{MFN2} cells would promote their clearance through the stimulation of autophagy/mitophagy. We observed a decrease in autophagosome formation leading to a reduction of the autophagy process initiation and consistent acceleration of cell division. Interestingly, we found that amongst the highest differentially expressed genes in CMT2A^{MFN2} fibroblasts, those controlling cell proliferation, extracellular matrix organization, and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR signalling pathway were mostly represented [8,9]. Based on this evidence and on the findings that mTORC2/AKT signalling pathway is highly elevated in MFN2 knocked-out cancer cells [11], we decided to verify whether mTORC2 activation was involved in the regulation of CMT2A^{MFN2} fibroblasts proliferation. In the present paper, we studied the AKT(Ser473) phosphorylation which is the target of the mTORC2 kinase activity [12-14] and consistently found that mTORC2-AKT signalling is activated in CMT2A^{MFN2} cells. We showed that treatment with torin1, a pharmacological and competitive inhibitor of mTOR, resulted in attenuation of CMT2A^{MFN2} fibroblasts proliferation rate, suggesting that this pathway is an important actor in CMT2A pathogenesis.

Materials and methods

Cell culture and reagents

Primary fibroblasts from a young patient affected by CMT2A^{MFN2} (c.650G>T/p.Cys217Phe) and a healthy control (individual with no histological or biochemical signs of mitochondrial disease), were obtained as reported in [8] after informed consent. Cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; EuroClone, ECB7501LX10) supplemented with 10% (v/v) fetal bovine serum (FBS; EuroClone, ECS5000L), 1% (v/v) L-glutamine (E EuroClone, ECB3000D), 1% (v/v) penicillin/streptomycin (EuroClone, ECB3001D), 50µg/ml of uridine (Sigma-Aldrich, U3003), in a humidified incubator at 37°*C and 5% CO2 avoiding confluence at any time.* All experiments were performed on cells with similar passage numbers, ranging from 5 to 8, to avoid any artefact due to senescence. For the experiments, growing cells were plated on sterile plastic dishes or flasks and allowed to adhere for at least 24 h before use. Torin1 (MedChemExpress, USA) was used at 0.1, 0.25 and 0.5 µM for 72 h, and DMSO as a vehicle.

Growth curves

CMT2A^{MFN2} fibroblasts and control cells were seeded in 24-well plates and grown for 3 days in presence of torin1 at 0.1, 0.25 and 0.5 µM and DMSO as vehicle-only treatment conditions. Cells were harvested by trypsinization and counted by hemocytometer every 24 h from day 1 to day 3. Cells were examined with Zeiss Primovert (Zeiss, Germany). A total of three individual experiments were performed.

Immunoblot analysis

For each treatment, fibroblasts grown on plates were collected at the confluence and homogenized in RIPA buffer (ThermoFisher Scientific, 89900) supplemented with proteases (Cell Signaling, 5871) and phosphatase (Cell Signaling, 5870S) inhibitors. The cells were sonicated on ice and centrifuged for 10 min at 16,000×g at 4°C and the protein concentrations were determined by Bradford assay (Bio-Rad, 500-0006). Thirty *µg* of cell proteins were lysed and denatured in Laemmli Buffer 2X (Bio-Rad, 1610737) separated by SDS-PAGE using homemade 10% separating gel and then transferred onto PVDF membranes using a Trans-Blot transfer apparatus (Bio-Rad, California, USA). The blocking agents used were 5% nonfat dry milk before overnight incubation with anti-phospho AKT (Ser473) and anti-GAPDH and Everyblot (Bio-Rad, 12010020) before overnight incubation with anti-total AKT antibodies.

Western blots were performed using primary anti-

bodies at the dilution of 1:1000 for anti-phospho AKT (Ser473) (Cell Signaling Technology D95), 1:2000 for anti-total AKT (Cell Signaling Technology, 40D4) and 1:15000 for anti-GAPDH (ProteinTech, 60004-1-Ig). Peroxidase Affinity Pure goat anti-mouse IgG and goat anti-rabbit IgG (Bio-Rad, 1706516 and 1706515, respectively) were added for 1 h at room temperature in the same buffer used for the primary antibodies. According to the manufacturer's instructions, reactive bands were detected using Clarity Western ECL Substrate (Bio-Rad, 1705061). Image acquisition was performed by the LI-COR C-Digit blot scanner and densitometric analysis was performed by the Image Studio Acquisition software (Licor, Lincoln, NE).

Statistical analysis

All statistical analyses were performed using PRISM® 7.04 in analytical software (GraphPad Software Inc, San Diego, CA) and Excel (Microsoft, Inc.). Results were expressed as average values ± SD of at least three independent determinations, each performed in triplicate, if not otherwise specified using CMT2A^{MFN2} versus sex and age-matched control fibroblasts. Statistical significance was calculated using Student's t parametric test set at: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$; and ****$p < 0.0001$. A one-way analysis of variance (ANOVA) test was performed to examine the differences between more than two dependent groups. The Bonferroni correction was used for multiple comparisons.

Results

Torin-1 restores CMT2A^{MFN2} fibroblasts' growth rate by decreasing AKT(Ser473) phosphorylation in a dose-dependent way

We have already demonstrated that inhibition of AKT activity with miransertib restores cell proliferation and autophagy in CMT2A^{MFN2} fibroblasts' [8,9]. To further dissect the mTOR/AKT signalling pathway involvement, we used a selective catalytic ATP-competitive mTOR inhibitor, *i.e.,* torin1, to reverse the effect of mTOR activation and prove that it is involved in the increase of cell proliferation rate of CMT2A^{MFN2} fibroblasts. We evaluated the antiproliferative activity of torin1 at 0.1, 0.25 and 0.5 μM for 72 h. We showed that the treatment caused a decrease in CMT2A^{MFN2} fibroblast growth rates compared to vehicle-only (DMSO)-treated cells as well as for the control fibroblasts according to the different inhibitor doses (Fig. 1). Since cell proliferation is controlled by mTORC2 through AKT(Ser473) phosphorylation [12-14], we evaluated mTORC2 activity by measuring the level of AKT-phosphorylation at Ser473 in CMT2A^{MFN2} fibroblasts. We found a very significant increase of AKT(Ser473) phosphorylation in basal conditions of CMT2A^{MFN2} compared to control fibroblasts. The inhibition by torin1 reduced AKT(Ser473) levels more strikingly in the mutant rather than in control fibroblasts. In detail, when we compared the levels of AKT(Ser473) at 0.5 uM torin1, we found no signal in mutant compared to control fibroblasts, despite the level of AKT(Ser473) in untreated conditions being much higher in mutant than in control. Furthermore, torin1 treatment was able to significantly reduce the abundance of AKT(Ser473) in a dose-dependent manner in CMT2A^{MFN2} fibroblasts (Fig. 2). The decreased cell proliferation rate reflected the different levels of AKT phosphorylation at Ser473. Taken together, these results suggested that the mTORC2 pathway is more activated in CMT2A^{MFN2} than in healthy control fibroblasts and highlighted the dependence of cell proliferation on this signalling pathway.

Discussion

The molecular mechanism by which MFN2 mutations lead to the disease and, importantly, how this mechanism can be tackled to modify CMT2A2's natural history is intensely studied [1,5-7,15-17]. Recently, our laboratory has shown that human CMT fibroblasts harbouring heterozygous single nucleotide substitution c.650G > T in MFN2, featured increased cell proliferation and downregulation of the autophagy process initiation. The transcriptomic analysis helped us to deep into the molecular pathways responsible for the dysfunctions found in CMT2A^{MFN2} fibroblasts. Most of the differentially expressed genes were enriched in cell population proliferation, extracellular matrix organization, and PI3K/AKT/mTOR signalling pathway. PI3K/mTOR/AKT signalling pathway has been proven to serve an important role in regulating cell proliferation, differentiation, autophagy, and apoptosis [18-22].

Based on this evidence, we showed that AKT activation is crucial in the regulation of proliferation in CMT2A^{MFN2} fibroblasts. Previously, we proved that the selective pharmacological inhibition of AKT with miransertib allowed for the restoration of the autophagy and cell proliferation rate in CMT2A^{MFN2} cells [8,9]. In the present study, we deepened the molecular mechanism responsible for the increased cell proliferation in CMT2A^{MFN2} fibroblasts focusing on mTORC2. We considered that mTORC2 mainly controls cell proliferation through the regulation of the phosphorylation *status* of AKT at Ser473 [12-14]. To this aim, we investigated AKT phosphorylation in CMT2A^{MFN2} cells, using torin1, a selective catalytic ATP-competitive mTOR inhibitor. Our results provide evidence of a strong increase of mTORC2-dependent phosphorylation of AKT(Ser473) in mutant fibroblasts. Torin1 treatment

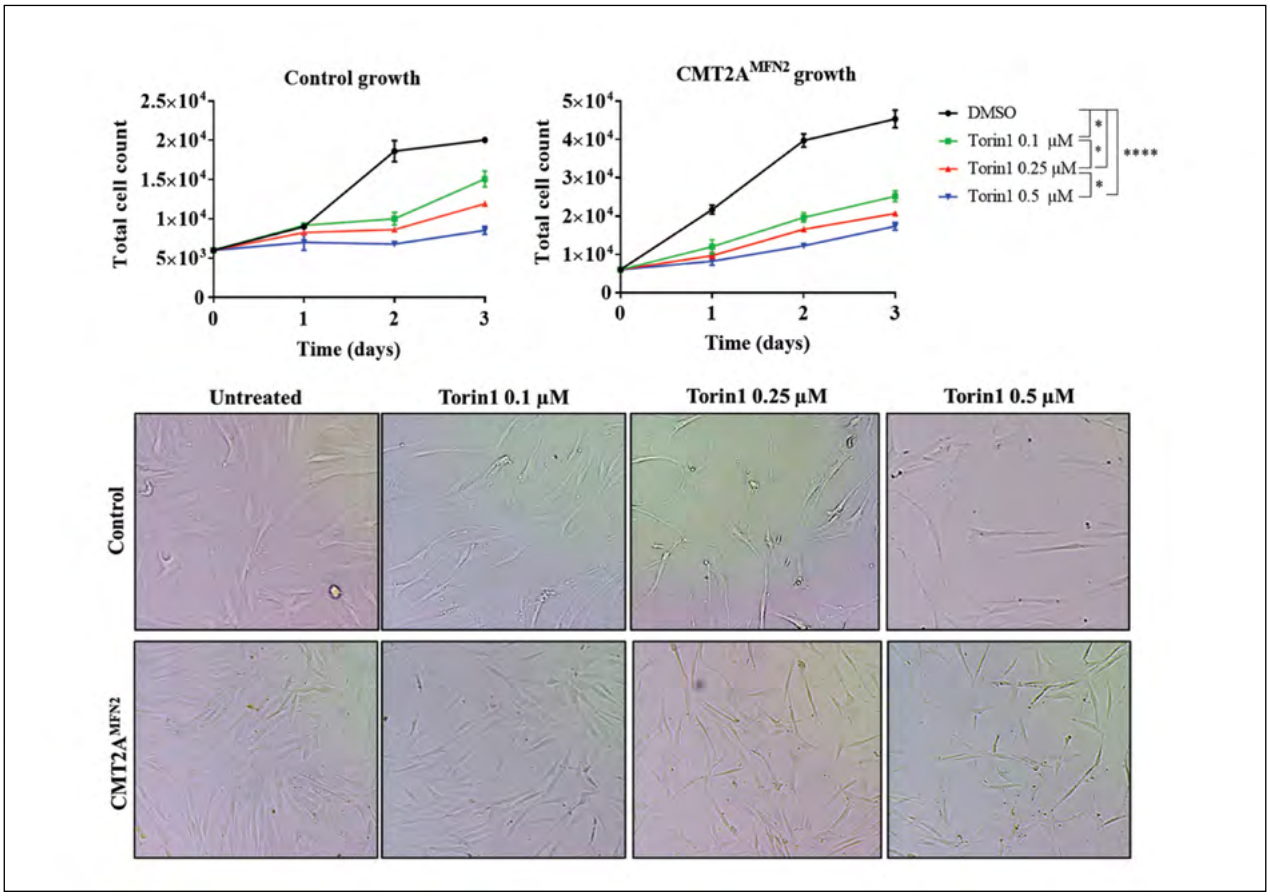

Figure 1. The cell growth rate of control and CMT2A^{MFN2} fibroblasts treated both with torin1 at 0.1, 0.25 and 0.5 µM and only vehicle (DMSO) for 72 h. Representative images of cells treated both with vehicle (DMSO) and torin1 at 0.1, 0.25 and 0.5 µM are shown. Data are presented as mean ± SD (n = 3). P-values refer to both control and CMT2A^{MFN2} fibroblasts. Student's t-test; $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. A one-way ANOVA test with Bonferroni's correction was performed for multiple comparisons.

showed anti-proliferative activity in CMT2A^{MFN2} cells by decreasing AKT(Ser473) phosphorylation in a dose-dependent manner. We derived that in agreement with the known anabolic effects of mTORC2/AKT pathway activation, the CMT2A^{MFN2} fibroblasts showed a remarkable increase in cell proliferation that can be reduced by the pharmacological targeting of mTORC2. This study reinforces our previous results confirming the involvement of the mTORC2/AKT pathway in CMT2A^{MFN2} disease. Acting on this pathway both miransertib and torin1 produce similar effects on cell proliferation. Herein, we showed that this pathway, extensively studied in cancer, can also be important in the pathogenesis of the neurodegenerative disease. PI3K/AKT/mTOR pathway is necessary to promote growth and proliferation over differentiation of adult stem cells, neural stem cells specifically [23]. It would be worth investigating the role of the PI3K/AKT/mTOR pathway in a cell system closely related to the disease, such as neuronal stem cells generated from CMT2A2 patients to understand if PI3K/AKT/mTOR signalling alterations could impact neural stem cell survival/differentiation. Overall, our results unveil mTORC2/AKT as novel potential targets that play a role in CMT2A2 pathophysiology.

Conclusions

In conclusion, our evidence showed that CMT2A^{MFN2} fibroblasts harbouring heterozygous single nucleotide substitution c.650G > T MFN2 showed increased proliferation because of mTORC2 activation. Considering that MFN2 is defined as a tumour suppressor, and based on our previous findings, we can hypothesize that MFN2 mutation may act as a dominant trait on cell proliferation, giving thus an unchecked trait on the cell division. Torin1 treatment can restore the cellular growth rates of

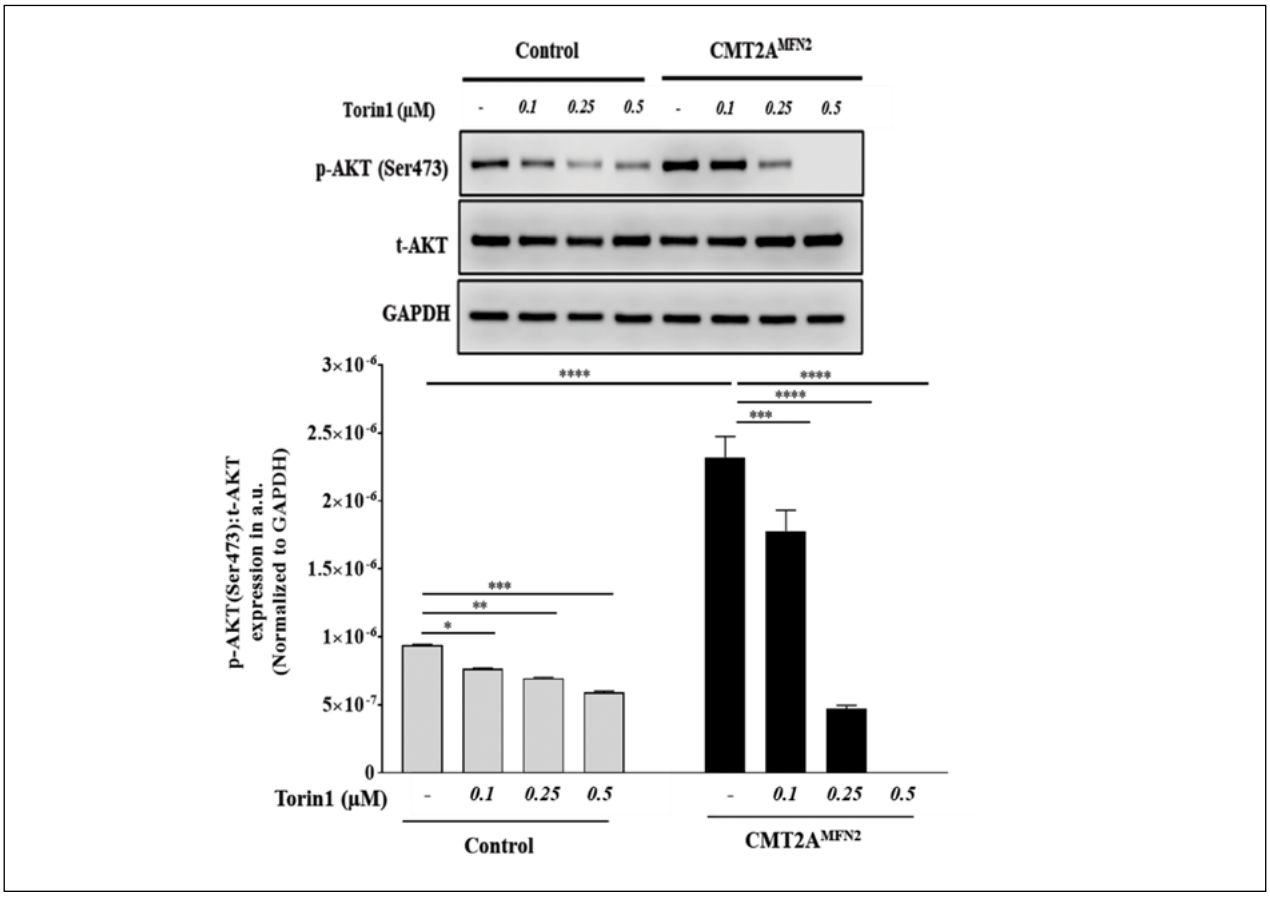

Figure 2. Representative western blot images of p-AKT(Ser473) and t-AKT in total cell lysates from CMT2A^{MFN2} and healthy control fibroblasts treated with torin1 at 0.1, 0.25 and 0.5 μM and vehicle-only (DMSO) for 72 h. Each signal was normalized to the GAPDH signal and densitometrical analysis of p-AKT(Ser473):t-AKT was performed. Data are presented as mean ± SD (n = 3). Student's t-test; $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. A one-way ANOVA test with Bonferroni's correction was performed for multiple comparisons.

CMT2A^{MFN2} fibroblasts in a dose dependent-manner acting on the AKT(Ser473) phosphorylation. Overall, these data established the dependence of cell proliferation on the mTORC2 pathway that thus represents a new potential actor in CMT2A2 pathophysiology.

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Conflict of interest statement

All authors have read and agreed to the published version of the manuscript. The Authors alone are responsible for the content and writing. No potential competing interest was reported by the authors.

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Author's contributions

PZ, VP: designed the research; FMS: provided the CMT2A^{MFN2} fibroblasts; PZ, AA, EAP: performed the research and analyzed the data; PZ, VP: wrote the manuscript.

Ethical consideration

As reported in ref. 8, the family's patient signed informed consent for research use of clinical data.

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