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Reduced expression of peroxisome-proliferator activated receptor gamma coactivator-1 α enhances α -synuclein oligomerization and down regulates AKT/GSK3 β signaling pathway in human neuronal cells that inducibly express α -synuclein

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Abstract

Intracellular accumulation of filamentous α -synuclein (α -Syn) aggregates to form Lewy bodies is a pathologic hallmark of Parkinson's disease. To determine whether mitochondrial impairment plays a role in accumulation of α -Syn oligomer, we used 3D5 cell culture model of human neuronal type whereby conditional overexpression of wild-type α -Syn via the tetracycline off (TetOff) induction mechanism results in formation of inclusions that exhibit many characteristics of Lewy bodies. In the present study, we compromised mitochondrial function in 3D5 cells by using shRNA to knockdown peroxisome-proliferator activated receptor gamma coactivator-1 α (PGC-1 α), a key regulator of mitochondrial biogenesis and cellular energy metabolism and found that PGC-1 α suppression at both protein and mRNA levels results in α -Syn accumulation (*i.e.* monomeric and oligomeric species in the TetOff-induced cells and monomeric only in the non-induced). These changes were accompanied with reduced mitochondrial potential as well as decreased levels of AKT, GSK3 β (total and Ser⁹-phosphorylated) and p53 that are important for cell survival. The extent to which these proteins decreased following PGC-1 α knockdown, in contrast to what was demonstrable with the viability assay, is greater in the induced than the non-induced. Together these findings indicate that such knockdown increases the propensity to accumulate α -Syn oligomers, but the accumulation appears to have very little toxic impact to the neuronal cells.

Parkinson's disease (PD) is characterized by selective loss of dopaminergic neurons in the substantia nigra and accumulation of α -Syn aggregates. A plethora of cellular processes including oxidative stress and mitochondrial dysfunction are considered to play a role in PD pathogenesis [19,23,14]. Overexpression of mutant α -Syn was reported to result in synaptic degeneration and neuronal cell death [9]. Neurotoxins that target mitochondrial complex I, such as MPTP/MPP⁺ and rotenone have been used in animal and cell culture studies to generate experimental models of PD [25,16,3]. PD cybrid cell line model has been successfully used to explore the contribution of mitochondrial dysfunction and mtDNA gene mutations to PD pathogenesis [21]. However, a recent report suggests that the axonal transport of mitochondria was significantly reduced in the process of PD cybrids and in rotenone-treated SH-SY5Y cells [4].

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Animal studies showed that knockout of PGC-1 α gene in mice can cause neurodegeneration [15]. In addition, such PGC-1 α knockout mice have increased sensitivity to oxidative stress mediators such as MPTP and kainic acid [20]. The PGC-1 α gene encodes a transcription factor that coordinates mitochondria biogenesis and augments the expression of several genes implicated to protect against oxidative damage. It has been suggested that regulation of cellular oxidative capacity through enhancing mitochondrial biogenesis could be beneficial for neuronal recovery and survival in (human) neurodegenerative disorders [6,11]. To date there is no information as to the consequence of suppressing mitochondrial biogenesis on α -Syn expression and aggregation. We addressed this issue by using lentivirus containing shRNAs-PGC-1 α to transduce human neuronal 3D5 cells that overexpress wild-type human α -Syn via the tetracycline-off (TetOff) inducible mechanism [10]. Such α -Syn overproduction results in intraneuronal accumulation of α -Syn aggregates that form filamentous inclusions resembling Lewy bodies. In the present study, the 3D5 cell were maintained in DMEM supplemented with 10% fetal bovine serum, 400 μ g/mL G418 (geneticin; EMD Biosciences), 1 μ g/mL puromycin (Sigma) and 2 μ g/mL tetracycline (Tet) and incubated at 37 °C with 5% CO₂. They were exposed 24 hours after seeding to 10 μ M retinoic acid (RA) in Neurobasal medium (Invitrogen) that is supplemented with B-27 supplement (Invitrogen), L-glutamine (2mM; Sigma), G418 (400 μ g/mL), puromycin (1 μ g/mL) and Tet (2 μ g/mL). On the 10th days of RA treatment, subsets of cultures were incubated with the aforementioned medium without Tet (Fig 1A). Cultures maintained in Tet-containing medium are referred as non-induced, whereas those without Tet are induced. After 2 or 5 days, all the cultures were re-seeded and maintained for additional 4 days prior to lentivirus transduction using shRNAs-PGC-1 α or non-target control (NT). Western blotting of cell lysates demonstrated a reduction of PGC-1 α following PGC-1 α knockdown (Figs 1B & C). The magnitude of such reduction is greater in the cultures with α -Syn-overexpression than those without (Figs 1D & E; 60% versus 40% and 70% versus 55% after TetOff induction for 9 and 14, respectively, days), suggesting a difference in transduction efficiency following α -Syn-overexpression. We confirmed, at the mRNA level by quantitative real-time-PCR analysis, effective suppression of PGC-1 α and demonstrated that the suppression has a greater impact on α -Syn-overexpressing cultures than their non-induced counterparts (Fig 1F). However, it remains to be determined how α -Syn-overexpression affects the efficiency of such viral transduction. It is plausible that overexpression of α -Syn may alter the susceptibility of the cells to viral infection because it is a lipid-binding protein after all. This scenario seems consistent with our finding that without such shRNA suppression, the level of PGC-1 α in the α -Syn-overexpressing cultures is comparable to that detectable with their non-induced counterparts. It is also possible that this may result from destabilization of the cytoskeleton, since recent studies have demonstrated interactions among α -Syn, actin and tau [8,18].

Western blotting also demonstrated that the cultures with PGC-1 α knockdown accumulate more monomeric α -Syn than their NT controls, regardless of whether they were induced or not (Figs 2A & B). However, high-molecular-weight α -Syn species, referred to as oligomers, appeared only in the induced. Most importantly, the level of such species was higher following PGC-1 α knockdown. The difference was greater in the cultures with 9-day induction than those derived from 14 days of induction (Figs 2C & D) but the cause for such discrepancy remains to be determined. Our quantitative real-time-PCR analysis showed that the level of α -Syn message is not affected by PGC-1 α knockdown (Fig 2E), indicating that other mechanisms are involved in the accumulation of monomeric α -Syn. In this regards, it is worth noting that exposure to neurotoxins such as MPTP, rotenone and others have been shown to heighten the level of monomeric α -Syn in cultured cells [17, 12].

To determine the consequence of PGC-1 α knockdown on cell viability and mitochondrial potential we used a fluorescence-based LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen), MitoTracker Red CMXRos and fluorescence microscopy. The results of viability assay clearly

showed reduced cell viability following PGC-1 α knockdown (Figs 3A & B). In comparison to the NT control fewer cells remained attached to the coverslip after PGC-1 α knockdown. Moreover, some of these cells displayed abnormal morphology (e.g. rounded cell bodies and retracted processes) indicating they are under stress (data not shown). The altered density and appearance were noted mainly after 3 days, but not less, of PGC-1 α knockdown. MitoTracker staining revealed that PGC-1 α knockdown results in weaker fluorescent signals (Figs 4A & B). Quantitative analysis of the cellular fluorescence from multiple fields showed that the α -Syn-overexpressing cells exhibit a significant 30% decrease ($p=0.0003$) in mitochondrial potential per cell following PGC-1 α knockdown ($n=165$ cells) when compared to their NT controls ($n=308$ cells) (Fig 4C). The results are consistent with those reported by others indicating that impaired mitochondrial biogenesis is detrimental to the cell survival [22, 13].

We next investigated whether PGC-1 α knockdown or α -Syn overexpression has any effects on GSK3 β and AKT. It has been reported that GSK3 β plays a role in regulating PGC-1 α protein stability [2]. Activation of AKT leads to phosphorylation of Ser⁹ and stabilization of PGC-1 α , whereas GSK3 β inhibition facilitates PGC-1 α degradation [5]. Results from our Western blot analysis demonstrated that the level of total AKT as well as that of total and Ser⁹ phosphorylated GSK3 β are lower in samples with PGC-1 α knockdown than NT controls (Figs 5A–5F). The changes, greater in the α -Syn-overexpressing cells than their non-induced counterparts, may reflect an attempt of these cells trying to stabilize their remaining PGC-1 α proteins. With respect to the relationship between α -Syn and GSK3 β expressions, a recent study showed that transgenic expression of α -Syn increases the level of GSK3 β [24]. It was also demonstrated in several experimental models of neurotoxin exposure that α -Syn is essential for GSK3 β activation and tau phosphorylation [7]. We have compared our NT controls with and without α -Syn overproduction and found that the induced tend to express more AKT and GSK3 β (total & Ser⁹ phosphorylated) although the difference is not statistically significant.

In our previous study we have demonstrated that α -Syn-overexpression has no impact on the viability of the 3D5 cells and on the levels of total phosphorylated p53, a tumor suppressor gene that is important for regulating cellular senescence [10]. However, it was reported by others that wild-type α -Syn transfected neuroblastoma cells have reduced levels of p53 when compared with that of their mock-transfected counterparts, and such responses were abolished after exposing the neuronal cultures to 6-hydroxydopamine [1]. To determine if p53 were involved in cell death observed in our PGC-1 α knockdown cultures we analyzed its expression at the protein level. Consistent with our previous studies, no significant difference in p53 level is evident between the non-induced and the induced of NT-transduced controls. Furthermore, we showed a decrease in the level of total p53 in the PGC-1 α -suppressed cultures suggesting that p53 is a downstream target of PGC-1 α (Figs 6A & B). Similar to that observed in our analysis of AKT, GSK3 β and PGC-1 α , the extent of p53 decrease caused by PGC-1 α knockdown was greater in the α -Syn-overexpressing cells than the non-induced. This is in contrast to the results obtained from our viability assays in which PGC-1 α knockdown led to a similar extent of decrease in the survival of both induced and non-induced cultures when compare to respective NT controls. Additional investigations are necessary to elucidate the molecular mechanism(s) contributing to the observed decrease of AKT, GSK3 β and p53. The lack of coordination between cell survival and the level of α -Syn oligomer raises the possibility that increased accumulation of α -Syn oligomer per se, as observed in our induced plus knockdown, is not cytotoxic. However, the long-term impact of α -Syn oligomerization in neuronal cells remains unclear. In this regard, it is worth noting that Parkinson's disease is associated with aging and its neurodegeneration proceeds over decades.

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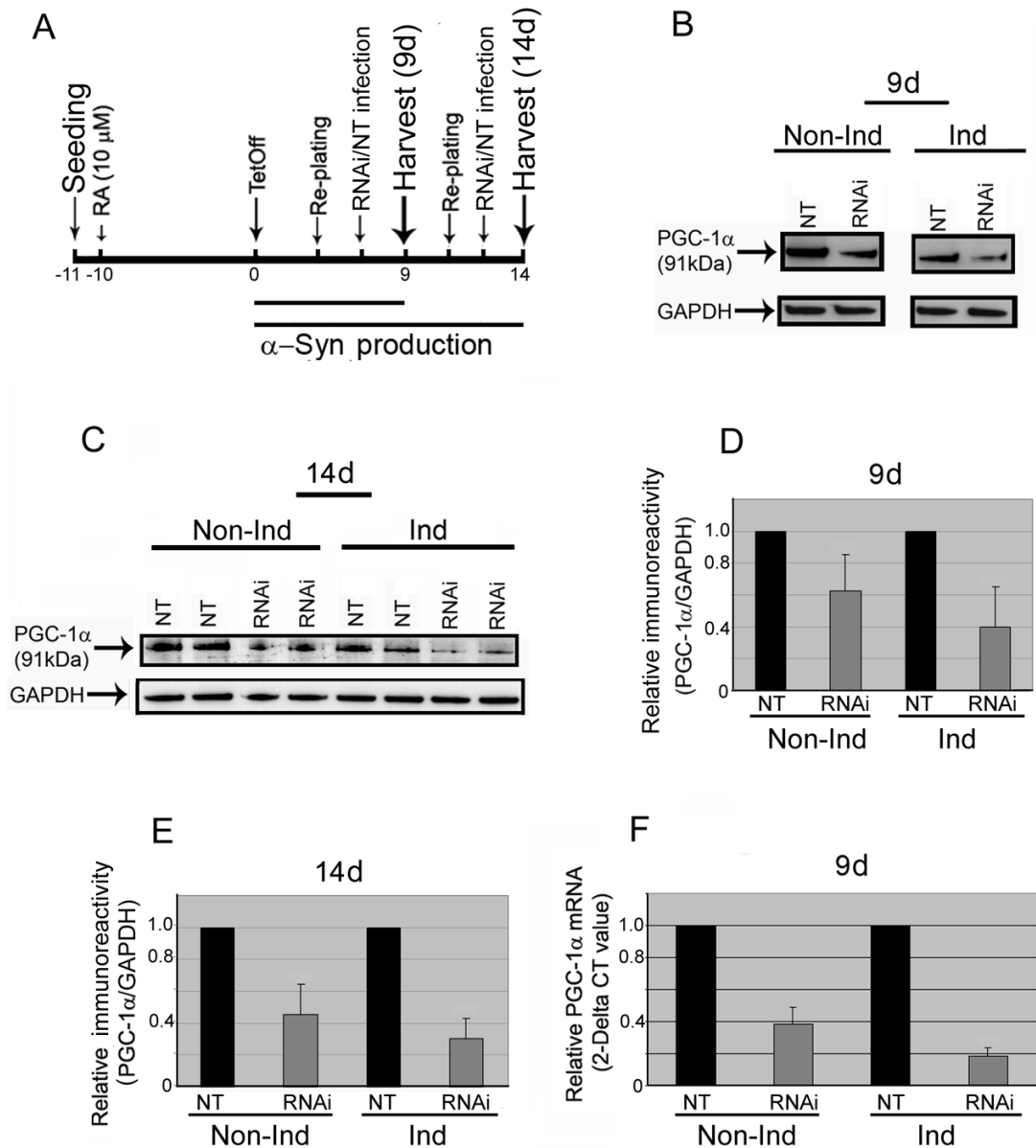


Fig. 1. Effect of PGC-1 α knockdown on differentiated 3D5 cells following TetOff induction

We undertook the culture paradigm illustrated in (A) to elicit α -Syn overexpression via TetOff induction for 9 and 14 days (9d & 14d) in 3D5 cells following their 10-day retinoic acid (RA) exposure (10 μ M) beginning on the day after seeding. (B) Western blot analyses of the differentiated, non-induced (Non-Ind) or TetOff-induced (Ind) 3D5 cultures infected with PGC-1 α shRNA-containing lentivirus (RNAi) or non-target control (NT), using GAPDH immunoreactivity to normalize sample loading, revealed that the RNAi strategy lowered PGC-1 α level effectively. As depicted in (D) the PGC-1 α knockdown of the 9-day induced cultures led to a significant reduction ($p < 0.0002$, $n = 6$) of PGC-1 α when compared to their sibling cultures transduced similarly using non-targeted controls. The RNAi treatment of their non-induced counterparts also reduced PGC-1 α significantly [$p < 0.005$, $n = 5$, error bar standard deviation (SD)], but the reduction is less than that observed in the induced. (C) Extension of α -Syn overexpression via TetOff induction to 14 days further augmented the susceptibility of differentiated 3D5 cells to PGC-1 α suppression by the RNAi ($p < 0.0001$, $n = 5$), (E) as compared

with their non-induced counterparts ($p < 0.001$, $n = 5$). Such increased sensitivity of the TetOff-induced cells to PGC-1 α knockdown was verified by quantitative real-time PCR and subsequent normalization with GAPDH at the mRNA level (F). Knockdown of the PGC-1 α gene using shRNA-containing lentivirus effectively reduced its mRNA level in the differentiated 3D5 cells after 9-day TetOff-induction (81% reduction as compared with the NT; $p = 0.02$, $n = 3$) but to a lesser extent in the non-induced (61% reduction; $p = 0.07$). The data are represented as means \pm SD of triplicate specimens that were derived from three experiments.

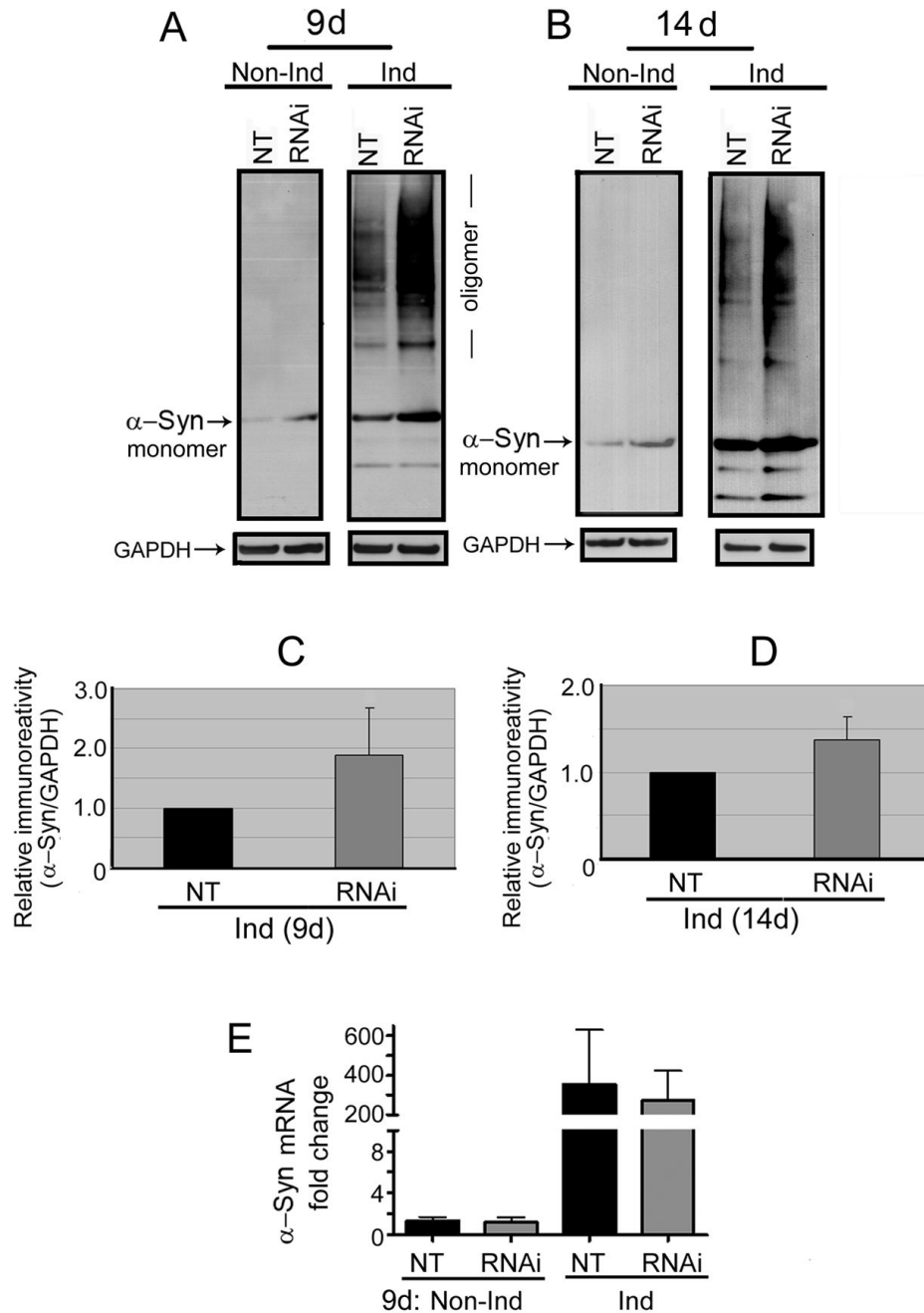


Fig. 2. PGC-1 α knockdown enhanced α -Syn aggregation

Following 9- (9d in A) or 14- (14d in B) day TetOff induction, transduction of RA-differentiated 3D5 cells using PGC-1 α shRNA-containing lentivirus resulted in augmented assembly of α -Syn oligomers/aggregates. Quantitative analyses of these blots revealed that TetOff induction for 9 (C) and 14 (D) days caused a 2- ($p=0.03$; $n=5$) and 1.5- ($p=0.03$; $n=5$), respectively, fold increase in α -Syn aggregation. (E) RT-PCR analyses revealed that PGC-1 α RNAi did not change the α -Syn message level which shows a 250–300 fold increase following TetOff induction. The mRNA quantification is normalized with GAPDH. The data are represented as means \pm SD of triplicate specimens that were derived from three experiments.

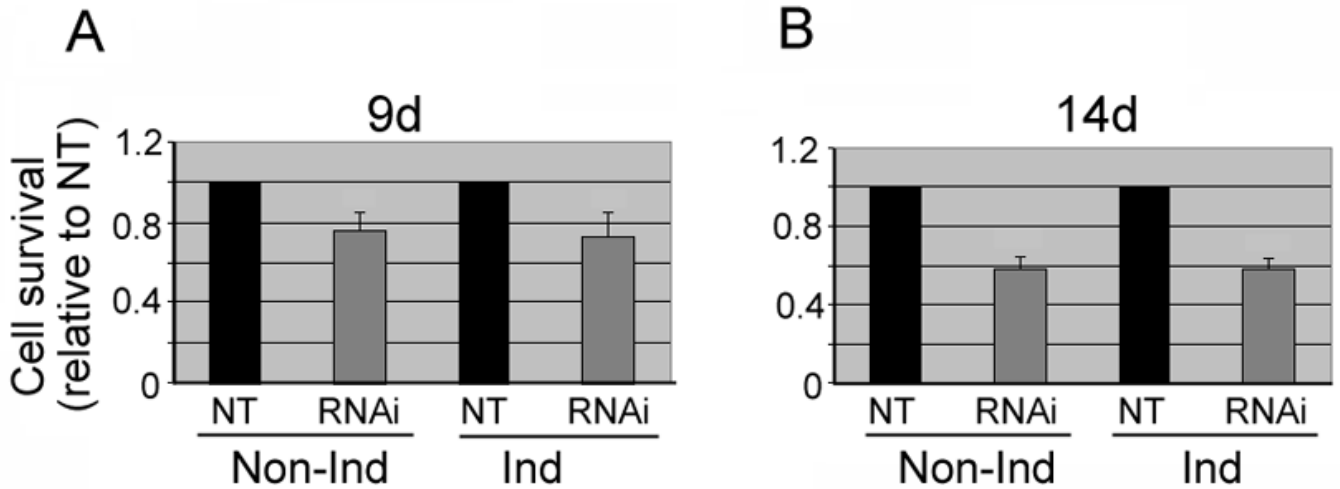


Fig. 3. Calcein AM Cytotoxicity assessment

To assess the viability of the replica 3D5 cultures transduced with PGC-1 α shRNA or its non-targeted controls, we used cell permeant calcein AM which is converted metabolically to fluorescent calcein thereby labeling live cells. (A) In comparison with their non-targeted controls (NT), the cells transduced after TetOff induction for 9 days showed reduced viability: 28% and 25% reduction in the induced and the non-induced, respectively, and (B) such reductions declined further (i.e. 41% and 42% for induced and non-induced, respectively) when α -Syn overproduction via TetOff induction was extended to 14 days. The data are represented as means \pm SD of 84 fields selected for each group from four experiments. Comparisons were made between RNAi, and NT-controls.

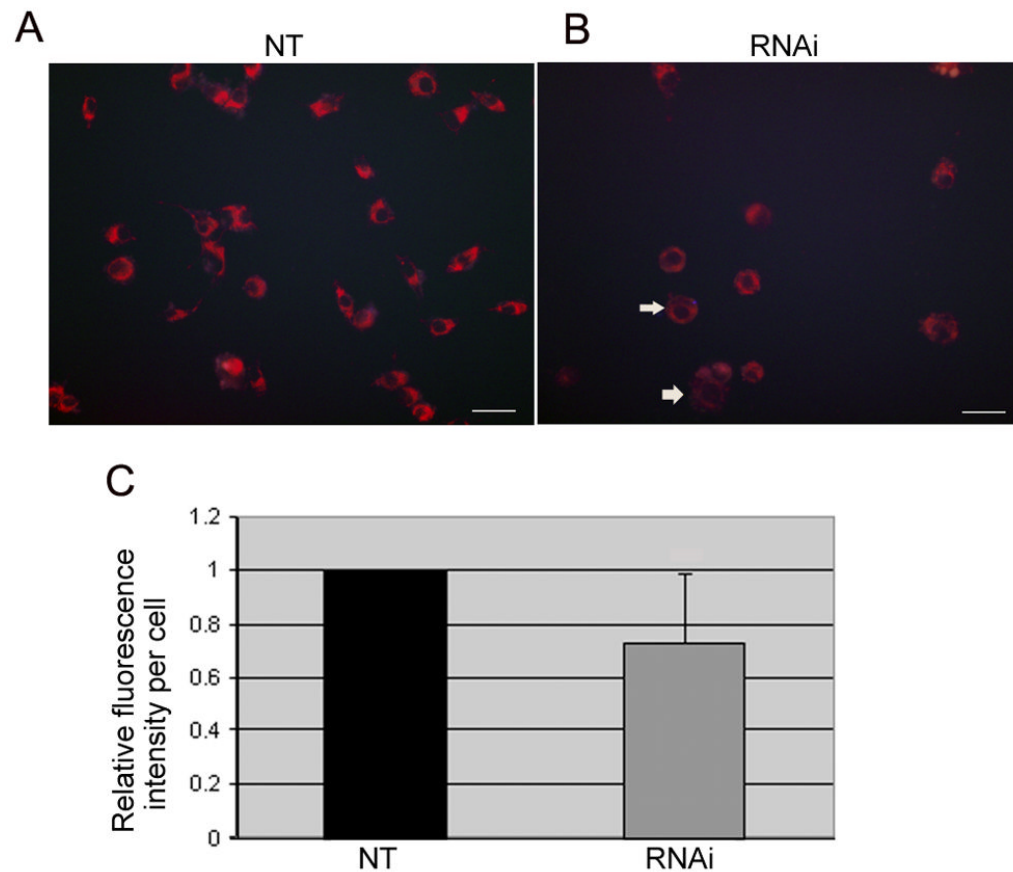


Fig. 4. Reduced mitochondrial membrane potential from PGC-1 α knockdown

We used MitoTracker Red CMXRos to probe the 3D5 cultures for the change in mitochondrial membrane potential (MMP) subsequent to their RNAi treatments. (A) Bright red fluorescent signal indicative of robust MMP was demonstrated in the non-target controls (NT). (B) In contrast, the fluorescent intensity appeared much lower in the RNAi-treated (arrows) suggesting compromised MMP. Quantitative analysis of the 3D5 cells using the MCID imaging software revealed that PGC-1 α knockdown leads to a significant reduction in MitoTracker fluorescence ($P=0.0003$, $n=165$ cells) in contrast with that of the NT-control ($n=308$ cells). Values are presented as means \pm SD.

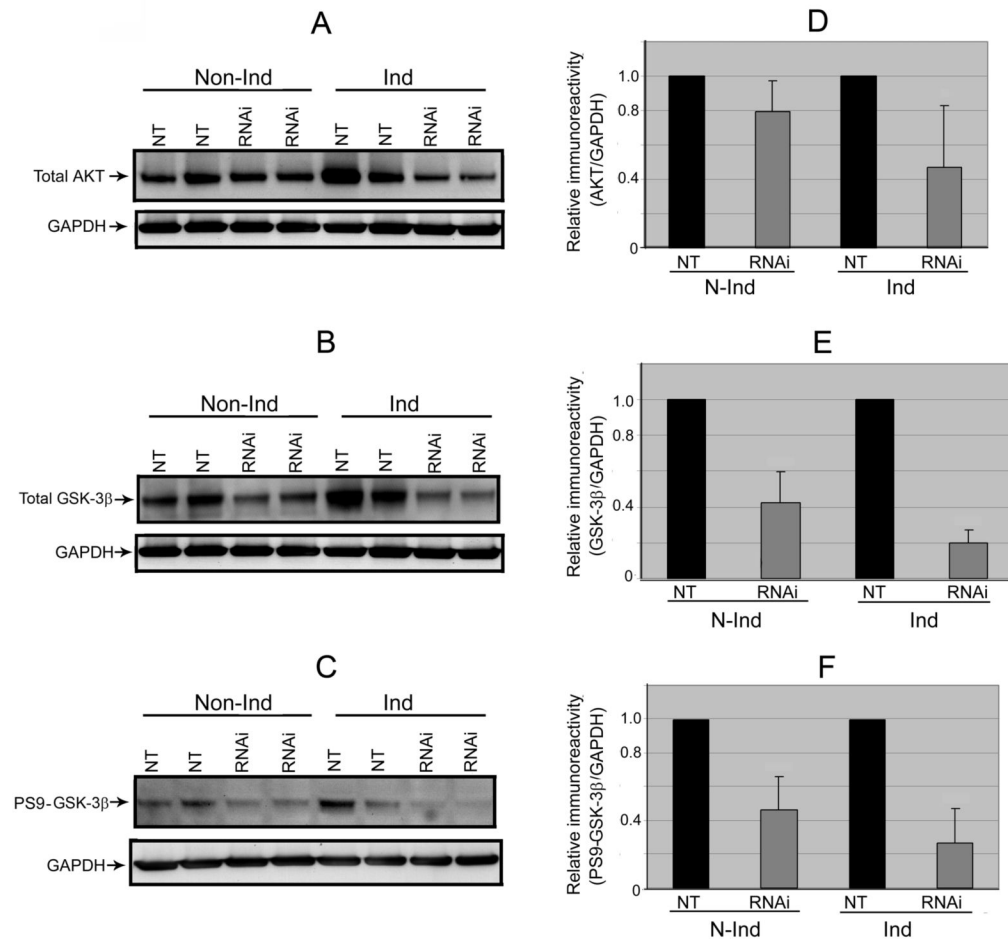


Fig. 5. Down-regulation of AKT, GSK-3 β and P-GSK-3 β -Ser⁹ from PGC-1 α RNAi

We probed lysate preparations of the differentiated 3D5 cultures, with or without TetOff induction, by immunoblotting analysis for (A) AKT and (B) GSK-3 β as well as (C) its phosphorylated form (p-GSK-3 β -Ser⁹). Immunoreactivities of AKT, GSK-3 β and p-GSK-3 β -Ser⁹ appeared down-regulated following PGC-1 α knockdown of non-induced cells and such reduction declined further after TetOff induction. This was verified quantitatively by densitometric analyses using GAPDH to normalize sample loading as illustrated for (D) AKT [20% reduction detectable with the non-induced ($p=0.06$) but 53% decrease after TetOff induction ($p=0.02$)], (E) GSK-3 β [57% drop discernible with the non-induced ($p<0.001$) while 80% decline in the induced ($p<0.0001$)] and (F) p-GSK-3 β -Ser⁹ [54% decrease detected in the non-induced but 74% reduction after TetOff induction ($p<0.001$)]. Values represent means \pm SD derived from immunoblots of the PGC-1 α RNAi-treated and their corresponding NT controls from 4 experiments.

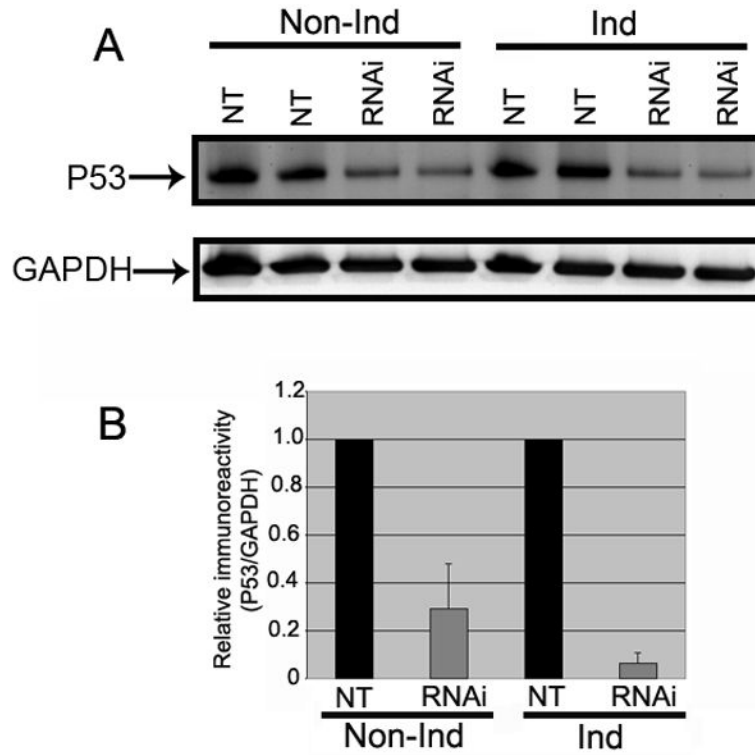


Fig. 6. Down-regulation of p53 in differentiated 3D5 cells upon PGC-1 α RNAi treatment

(A) Cell lysates were probed with p53 antibodies. The p53 protein expression was lowered in PGC-1 α RNAi treated cells than Non-targeted controls (NT) in both induced and non-induced conditions. (B) Quantitative analysis of p53 Western blots demonstrated that the p53 level was significantly decreased in PGC-1 α RNAi treated induced groups (n=4) than non-targeted controls (n=4). Induced groups: (90% reduction, $p < 0.0001$); Non-induced groups: (71% reduction, $p < 0.001$). All data were normalized using GAPDH. Values are represented as means \pm SD.