SIRT1 Silencing Confers Neuroprotection Through IGF-1 Pathway Activation

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The following study demonstrated that, in in vitro differentiated neurons, SIRT1 silencing induced an increase of IGF-1 protein expression and secretion and of IGF-1R protein levels which, in turn, prolonged neuronal cell survival in presence of an apoptotic insult. On the contrary, SIRT1 overexpression increased cell death. In particular, IGF-1 and IGF-1R expression levels were negatively regulated by SIRT1. In SIRT1 silenced cells, the increase in IGF-1 and IGF-1R expression was associated to an increase in AKT and ERK1/2 phosphorylation. Moreover, neuronal differentiation was reduced in SIRT1 overexpressing cells and increased in SIRT1 silenced cells. We conclude that SIRT1 silenced neurons appear more committed to differentiation and more resistant to cell death through the activation of IGF-1 survival pathway.
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SIRT1 is a member of the *SIR2*-like gene family that encodes for sirtuins, a class III histone deacetylases and/or mono-ADP-ribosyltransferases. Sirtuins affect many metabolic and stress resistance pathways (Guarente, 2011). In mammals, there are seven sirtuins (SIRT1 to SIRT7) with different functions and different cellular localization (Guarente, 2011). By using nicotinamide adenine dinucleotide (NAD^+) as their activating cofactor, they are able to sense energy availability, circadian rhythms and cellular stresses. Sirtuins respond to metabolic or toxic stresses by deacetylating several cellular factors. SIRT1 deacetylates many histonic and non histonic proteins such as p53 (Vaziri et al., 2001; Langley et al., 2002), the forkhead transcription factors (FOXOs; Motta et al., 2004), nuclear factor kappa-light-chain (NF-kB; Yeung et al., 2004) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; Nemoto et al., 2005). SIRT1 is also involved in neuronal degeneration. SIRT1 can be neuroprotective or neurotoxic depending on conditions, cellular stress and cellular type. SIRT1 has been shown to be protective against neuronal apoptosis in cerebellar granule neurons (Pfister et al., 2008), in the axonal Wallerian degeneration model (Araki et al., 2004), in Alzheimer disease (AD) models (Qin et al., 2006; Kim et al., 2007) and in cortical neurons (Hasegawa and Yoshikawa, 2008). In addition, SIRT1 improves learning and memory by activating the brain-derived neurotrophic factor (BDNF) gene (Gao et al., 2010). SIRT1 neurotoxicity has been also documented. SIRT1

overexpression in mice induced a memory deficit, and had no neuroprotective effects against damage induced by ischemia or by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Kakefuda et al., 2009). Moreover, caloric restriction (CR) causes both SIRT1 increased expression in some regions of the brain (such as the hypothalamus), and SIRT1 downregulation in others (Chen et al., 2008; Liu et al., 2008). In addition, SIRT1 chemical inactivation has been shown to be beneficial in neurons (Chong et al., 2005; Holland et al., 2008; Liu et al., 2008, 2009; Tang, 2010).
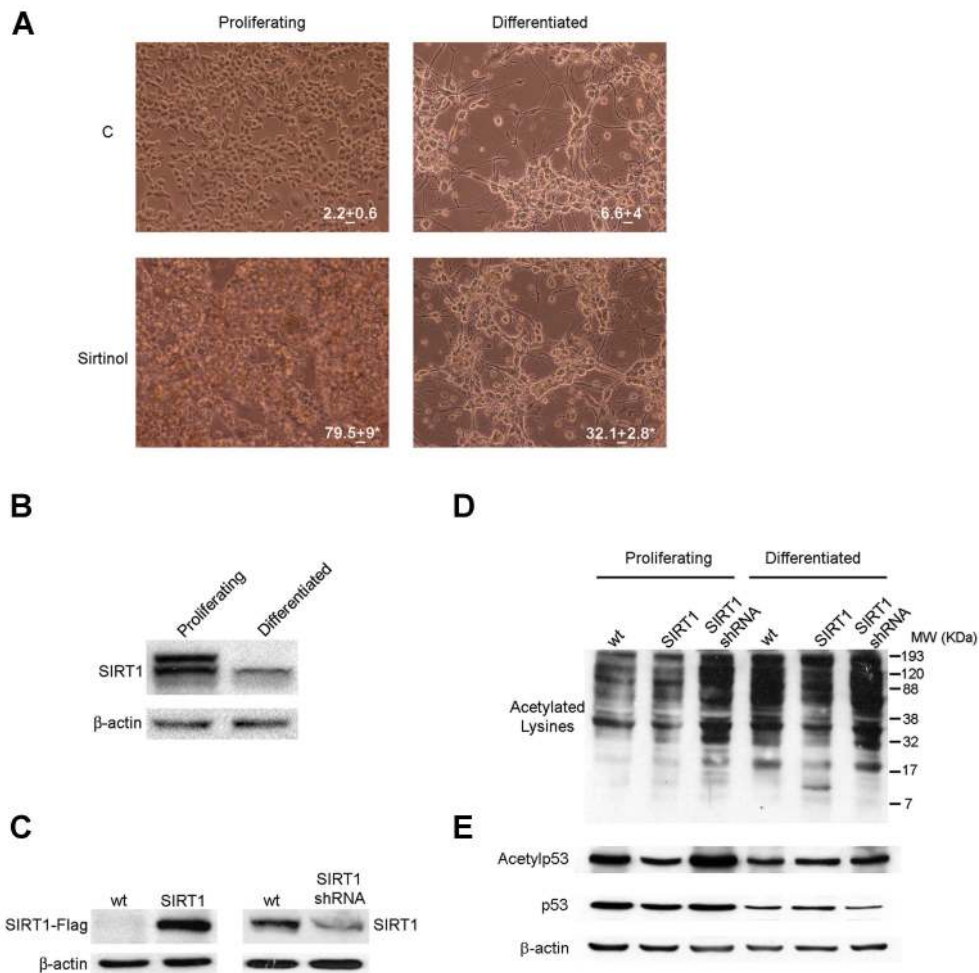
Fig. 1. SIRT1 modulation in NG108-15 cells. A: Reduced sensitivity of NaB differentiated NG108-15 cells to Sirtinol. Proliferating and NaB differentiated cells were either left untreated or treated with 50 μM Sirtinol for 24 h. Morphology was evaluated by phase contrast microscopy. Pictures were taken at 20× with a digital camera on an inverted microscope. Percentage of cell death indicated in each image was calculated by flow cytometry analysis of three independent experiments. C = control untreated cells. *, $P < 0.05$. B: Decreased SIRT1 expression in NaB differentiated NG108-15 cells. Proliferating and NaB differentiated cells were lysed and SIRT1 levels measured by Western Blot. β-actin was used as loading control. C: SIRT1 overexpression and silencing in NG108-15 cells. Stable clones overexpressing Flag-tagged SIRT1 were obtained by transfecting NG108-15 cells with a pcDNA3.1-FlagSIRT1 as indicated in Materials and Methods Section. At the same time, stable clones silenced for SIRT1 were obtained by transducing NG-108-15 cells with lentiviral particles expressing SIRT1-shRNA as indicated under Materials and Methods Section. SIRT1 overexpression was measured with an anti-Flag antibody (left side). SIRT1 silencing was measured with an anti-SIRT1 antibody (right side). β-actin was used as loading control. D: Global protein lysine acetylation levels in SIRT1 overexpressing and silenced NG108-15 cells. Proliferating and NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were lysed and global acetylation state of lysines was measured by Western blot. Results are representative of three independent experiments. E: p53 acetylation levels in wt, SIRT1 overexpressing and SIRT1 silenced NG108-15 cells. Proliferating and NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were lysed and p53 acetylation state was measured by Western Blot as described in Materials and Methods Section. Results are representative of three independent experiments.

Insuline like growth factor-1 (IGF-1) affects cellular survival, metabolism and glucose homeostasis by activating the Ser/Thr-kinase B/AKT pathway (Laviola et al., 2007). IGF-1 plays also a role in the central nervous system (Broughton and Partridge, 2009). In the brain IGF-1 promotes neuron survival, neurite outgrowth, maturation of oligodendrocytes, myelination (D'Ercole et al., 1996), and improves learning and memory (Van der Heide et al., 2006). Alteration of IGF-1 protein expression has been associated with neurodegenerative pathologies (Trejo et al., 2004) and with cognitive decline during aging (Markowska et al., 1998). In addition, IGF-1 has been shown to posses neuroprotective and neurogenic function during ischemic brain injury (Guan et al., 2001, 2003). IGF-1 acts as neuroprotective factor by inducing NF-kB via PI 3-kinase pathway (Heck et al., 1999).

Previously, we observed that neuronal differentiated NG108-15 cells are more resistant to STS-induced apoptosis (Pucci et al., 2008). The acquired resistance of the differentiated cellular phenotype depends on an increased IGF-1 expression/secretion and consequent activation of survival pathways (Pucci et al., 2008). Several authors have highlighted the connection between SIRT1 and IGF-1 pathways (Bordone et al., 2006; Li et al., 2008; Longo, 2009). Therefore, we hypothesized a role for SIRT1 in regulating IGF-1 survival pathway in in vitro differentiated neurons.

In the following study, we investigated the interplay between SIRT1 and IGF-1 pathways in neuronal cell death. SIRT1 protein level modulation in in vitro differentiated NG108-15 cells altered neuronal differentiation and resistance to several death stimuli. In particular, SIRT1 silencing activated IGF-1 pathway by

increasing IGF-1 secretion and IGF-1R expression levels. SIRT1 silenced neurons appeared, therefore, more committed to differentiation and more resistant to cell death through the activation of IGF-1 survival pathway.

Materials and Methods
Materials

Unless stated otherwise reagents were purchased from Sigma–Aldrich (St. Louis, MO). Sorbitol was dissolved directly in cell culture medium as 1 mM solution. Staurosporine (STS) was dissolved in dimethyl sulfoxide (DMSO) as 1 mM stock solution. Sirtinol was dissolved in DMSO as 25 mM stock solution. Camptothecin was dissolved in DMSO as 25 mM stock solution. Thapsigargin was dissolved in DMSO as 1 mM stock solution. IGF-1 was dissolved as 50 ng/ml solution in H_2O. Sodium butyrate (NaB) was dissolved as 0.5 M solution in H_2O. Propidium Iodide (PI) was dissolved as 100 μg/ml in H_2O. Collagen I was dissolved as 100 μg/ml solution in H_2O. Dulbecco's modified Eagle's medium (DMEM) without pyruvate and HAT supplement were purchased from Invitrogen Life Technologies (Carlsbad, CA). FBS was purchased from Sigma–Aldrich. Anti-Actina-β polyclonal antibody was purchased from Spring Bioscience (Pleasanton, CA). Anti MAP51B-MAP5 monoclonal antibody was purchased from GENETEX (San Antonio, TX). Anti IGF-1 monoclonal antibody was purchased from ABcam (Cambridge, UK). Anti-IGF-1-receptor (IGF-1R)-β and anti acetyl-p53 (lys379) polyclonal antibodies were purchased from Cell Signalling Technology, Inc. (Danvers, MA). Anti Acetylated Lysine polyclonal antibody, HRP Conjugate was purchased from Assay Designs (Ann Arbor, MI). Anti-Akt polyclonal antibody, anti-phospho-Akt polyclonal antibody (Thr308), anti-ERK1/2 polyclonal antibody and anti-phospho-ERK1/2 polyclonal antibody (Tyr202/Thr204) were purchased from Invitrogen (Milan, Italy). Anti-Sirt1 polyclonal antibody and Anti-Tubulin monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An enhanced chemiluminescent detection system (ECL kit) was purchased from Euroclone (Milan, Italy).

Cell culture

The cell line NG108-15 (mouse neuroblastoma/rat glioma hybrid cell line) was purchased from ATCC (Manassas, VA) and grown in DMEM medium supplemented with 10% (v/v) FBS and HAT supplement. For differentiation experiments, cells were plated on collagen I on plastic dishes (100 mm × 20 mm) and cultured in DMEM without pyruvate supplemented with 2% FBS for 5 days in presence of 1 mM NaB. Medium was changed every other day. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Stable transfections

Sirt1 cDNA clone was purchased from Origene Company (Catalog No: RC218134-20). Transfections were performed with *Trans*IT-Neural Transfection Reagent (MIRUS, Madison, WI) according to the manufacturer's protocols. Stable overexpressing cell lines were selected by adding 350 μg/ml of Geneticin G-418 Sulphate (Invitrogen, Paisley, UK) to the fresh complete medium.

Lentiviral transduction

Mission[TM] TRC shRNA lentiviral transduction particles expressing short hairpin RNA (shRNA) targeting SIRT1 was purchased from Sigma–Aldrich. Stably transduced clones were generated according to the manufacturer's instructions. Briefly, cells were seeded on a 24-well plate. The following day cells were infected. After 24 h medium was changed. Selection of stable clones was started 24 h later with the addition of 3 μg/ml of puromycin.

Microscopy

Cellular morphology was evaluated in proliferating and differentiated cells after 50 μM Sirtinol treatment for 24 h by phase contrast microscopy without preliminary fixation. Pictures were produced using an inverted microscope (NIKON Eclipse TE2000U) and a digital camera (NIKON DS5Mc).

Western blotting

Cells were harvested, washed twice in PBS and resuspended in a volume of lysis buffer (50 mM Tris pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). After 30 min on ice, lysates were centrifuged and protein collected. Protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Proteins were normalized to 100 μg/lane and applied to SDS–polyacrylamide gels. The gels were blotted (1:30 h at 230 mA) onto a Hybond-ECL nitrocellulose filter (Amersham Life science, Inc., Arlington Heights, IL). A Kaleidoscope prestained protein solution (Bio-Rad Laboratories) was used as a molecular weight standard. The filter was washed twice with TBS-0.1% Tween-20 buffer (TBS-T), before blocking non-specific binding sites with 5% milk /TBS-T for 1 h. The filter then was incubated for 1 h at room temperature with the specific antibody diluted in 3% milk/TBS-T. The nitrocellulose filter was washed twice and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit. Detection was performed at room temperature using the ECL method.

Flow cytometry

For proliferative studies, cells (5×10^5) were plated in 10 mm dishes. The following day the cells were treated with 0.5 μM STS, 7.5 μM thapsigargin, 1 M sorbitol and 25 μM camptothecin for the times indicated in Figures 2C and 3. After the treatment, the cells were harvested by centrifugation (10 min at 1,600 rpm at 4°C), washed with 5 ml PBS, and resuspended in 500 μl PBS to which 5 ml of cold 70% EtOH was slowly added while stirring. Following overnight incubation at 4°C, cells were centrifuged at 1,600 rpm for 5 min at 4°C and washed once with PBS. The cells were then resuspended in 500 μl of a solution composed by 50 μg/ml PI, 250 μg/ml RNase A and PBS 1× and kept at 37°C for 30 min. DNA content was analyzed on a COULTER EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA).

Statistical analysis

All experiments were repeated 3–5 times and the mean and the standard error of the mean [SEM] were determined. Significant differences between sets of values for control and test groups were assessed by using Student's *t*-test. A *P*-value refers to a comparison of a measured parameter in the experimental group with that of the appropriate control; significance was set at $P < 0.05$.

Results
Differentiated NG108-15 neurons are resistant to Sirtinol treatment and express less SIRT1 than NG108-15 cells

To test SIRT1 role in neuronal survival, we tested the toxicity of the SIRT1 inhibitor sirtinol, on NG108-15 cells. Cultures were grown in proliferating or differentiating conditions. Cells were either left untreated (C) or treated with 50 μM sirtinol for 24 h. Figure 1A shows light microscope pictures of treated and untreated cells. Cell death percentage was measured by flow cytometry. Sirtinol treatment killed about 79.5% of proliferating cells. By contrast, only 32.1% of cell death was measured in neuronal differentiated NG108-15 cells exposed to sirtinol.

We hypothesized that sirtinol resistance of NG108-15 differentiated cells could be due to a reduced expression of SIRT1. Therefore, we measured SIRT1 expression in in vitro

Fig. 3. SIRT1 effects on cell death induced by thapsigargin, camptothecin, and sorbitol. Proliferating and NaB differentiated wt and SIRT1 overexpressing cells were either left untreated or treated with thapsigargin, camptothecin, or sorbitol for the indicated times. Percentage of cell death was measured by flow cytometry analysis, as described in Materials and Methods Section. C = control untreated cells. *, significantly different from thapsigargin treated wt cells. ^, significantly different from sorbitol treated wt cells. #, significantly different from camptothecin treated wt cells. Significance was set at $P < 0.05$. Error bars represent standard deviation (SD). Results in each part are the average of three independent experiments.
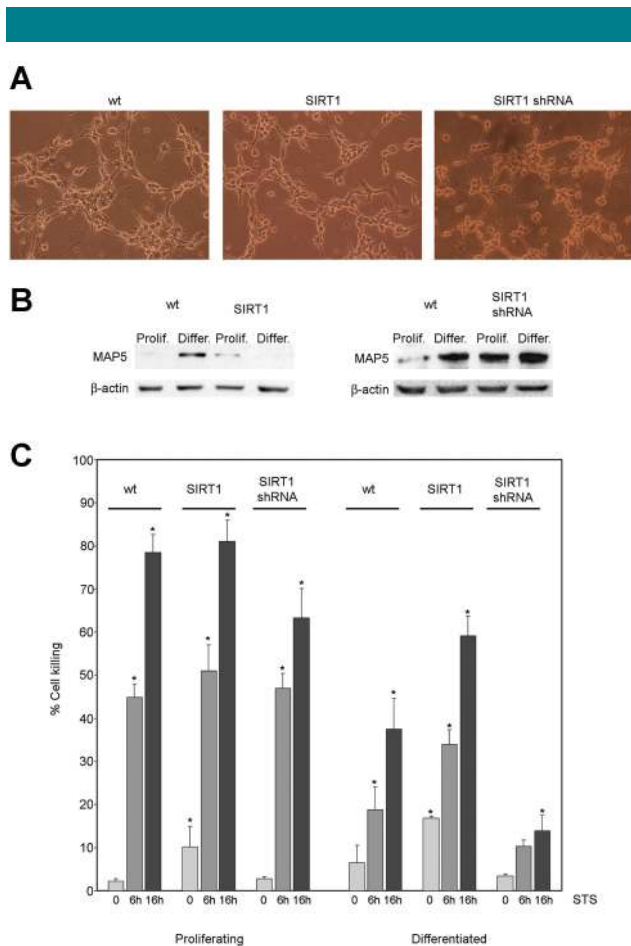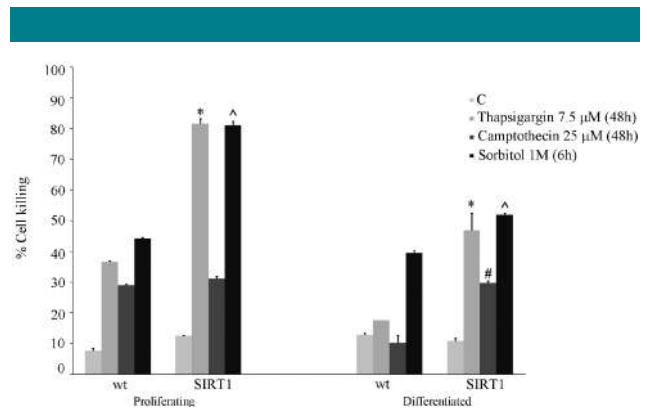
Fig. 2. SIRT1 effects on both neuronal differentiation and STS-induced cell death in NG108-15 cells. A: Wt, SIRT1 overexpressing and SIRT1 silenced cells were NaB differentiated. Morphology was evaluated in differentiated cells by phase contrast microscopy. Pictures were taken at 20× with a digital camera on an inverted microscope. B: Proliferating and NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were lysed and differentiation measured by MAP5 expression as reported in Material and Methods Section. β-actin was used as loading control. C: Proliferating and NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were either left untreated or treated with STS for the indicated times. Percentage of cell death was measured by flow cytometry analysis, as described in Materials and Methods Section. C = control untreated cells. *, $P < 0.05$. Error bars represent standard deviation (SD). Results in each part are the average of three independent experiments.

neuronal differentiated cells. As Figure 1B shows, SIRT1 levels decreased in differentiated NG108-15 cells compared to their proliferating counterpart.

SIRT1 overexpression and silencing in NG108-15 cells affects cellular differentiation and stress response

To assess the role of SIRT1 in cellular differentiation and stress resistance, a clone of NG108-15 cells stably overexpressing SIRT1 (clone 8) as well as a clone in which SIRT1 was stably silenced (clone 645) by shRNA transduction were produced. SIRT1 levels in overexpressing and silenced SIRT1 clones are shown in Figure 1C.

To confirm SIRT1 overexpression and silencing, we measured global protein lysine acetylation cellular levels in wt as

well as in overexpressing and silenced SIRT1 clones. As Figure 1D (lanes 2 and 5) shows, cellular acetylation was decreased both in proliferating and differentiated SIRT1 overexpressing cells compared to the wt NG108-15 cells. By contrast, both proliferating and differentiated SIRT1 silenced cells showed a higher level of total protein acetylation (Fig. 1D lanes 3 and 6). To further confirm that SIRT1 expression manipulation resulted in an increased or decreased SIRT1 deacetylation activity, the acetylation state of p53 was measured in wt, SIRT1 overexpressing and SIRT1 silenced cells in proliferating and differentiating culture conditions (Fig. 1E). As expected p53 acetylation was decreased in SIRT1 overexpressing cells and increased in SIRT1 silenced cells comparing to wt cells.

Since we have observed a decrease in SIRT1 levels after NG108-15 differentiation, we decided to study if also the opposite was true, that is if NG108-15 differentiation could be influenced by increasing or decreasing SIRT1 levels. Figure 2A shows that the typical neuronal network of wt NG108-15 differentiated cells (left part) was reduced in SIRT1 overexpressing clones (middle part) and increased in SIRT1 silenced cells (right part). NG108-15 differentiation was also measured through the expression of the neuronal marker MAP5. Figure 2B shows that MAP5 expression levels were reduced in SIRT1 overexpressing cells and increased in SIRT1 silenced cells. Notably, MAP5 was abundantly expressed in proliferating SIRT1 silenced cells (Fig. 2B).

To study SIRT1 involvement in neuronal stress resistance, cell death induced by several stimuli was measured in wt, SIRT1 overexpressing and SIRT1 silenced NG108-15 cells. Figure 2C shows that, as previously observed by us (Pucci et al., 2008), differentiation of wt NG108-15 cells increased resistance to 0.5 μM STS with a percentage of cell killing of 18% after 6 h and 37% after 16 h treatment. Overexpression of SIRT1 reduced such resistance to STS. STS killed 35% after 6 h and 57% after 16 h of differentiated SIRT1 overexpressing cells. On the other hand, SIRT1 silencing increased resistance to STS cell death after differentiation with only 8% after 6 h and 12% after 16 h of cells being killed (Fig. 2C). Importantly, SIRT1

silencing significantly reduced cell killing of proliferating cells. In fact, 16 h STS treatment killed about 80% of wt and SIRT1 overexpressing cells and only 63.4% of SIRT1 silenced cells (Fig. 2C).

To rule out the possibility that the results described above were restricted to the particular selected clones, neuronal viability after STS treatment was also studied using additional SIRT1 overexpressing and SIRT1 silenced NG108-15 clones. Supplementary Figure 1 shows that the analysis of two different SIRT1 overexpressing (clones 7 and 9) and one SIRT1 silenced clone (clone 646) produced comparable results in terms of cell viability.

Sirt1 effect on neuronal viability was independent of cell death stimulus. Cell death was measured by flow cytometry after treatment with 7.5 μM thapsigargin (48 h), 1 M sorbitol (6 h), and 25 μM camptothecin (48 h). As Figure 3 shows differentiated cells were in general more resistant to cell death. Importantly, SIRT1 overexpressing, proliferating and differentiated, cells were more sensitive than wt cells to thapsigargin and sorbitol. Camptothecin-induced cell death increased only in differentiated SIRT1 overexpressing cells.

SIRT1 effect on cell cycle was also investigated. SIRT1 overexpression induced a statistically significant decrease of G0/G1 percentage in proliferating cells, compared to WT cells (Fig. S2). Also in differentiated cells SIRT1 overexpression induced a statistically significant reduction of G0/G1 cells, associated to an increase in the percentage of cells in S phase (Fig. S2). No effect on cell cycle was observed when SIRT1 was silenced.

IGF-1 and IGF-1R expression levels are regulated by SIRT1

Our data showed a role for IGF-1 in inducing cell death resistance (Pucci et al., 2008), and a negative effect of SIRT1 in cellular resistance (Figs. 2C and 3). Therefore, we hypothesized

an interplay between IGF-1 and SIRT1 pathways to regulate neuron resistance. To pursue this goal, secreted and intracellular IGF-1 levels were measured in wt, SIRT1 overexpressing and SIRT1 silenced cells. Figure 4A shows that IGF-1 secretion was increased by STS treatment in wt NG108-15 cells after 16 h of STS treatment. The increase is particularly evident in differentiated cells. Differentiated SIRT1 silenced cells secreted IGF-1 in the absence of STS treatment. The presence of STS increased such release both after 6 and 16 h (Fig. 4A). On the contrary, SIRT1 overexpressing cells showed a reduced IGF-1 secretion after STS treatment in proliferating cells compared to wt cells (Fig. 4A). In differentiated SIRT1 overexpressing cells IGF-1 secretion was induced by STS only after 16 h of treatment (Fig. 4A). Intracellular IGF-1 was also measured. Interestingly, SIRT1 silenced cells showed a significant increase in IGF-1 intracellular expression after STS treatment compared to wt cells. Moreover, both proliferating and differentiated SIRT1 silenced cells had an increased basal expression of intracellular IGF-1 compared to wt and SIRT1 overexpressing cells (Fig. 4B). On the contrary, SIRT1 overexpressing cells had a decreased expression of intracellular IGF-1 compared to wt and to SIRT1 silenced cells after STS treatments (Fig. 4B). Since activation of the IGF-1 pathway depends also on IGF-1R expression, IGF-1R protein levels were measured in proliferating and differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells in the presence or absence of STS. Figure 5A shows that proliferating SIRT1 silenced cells express more IGF-1R compared to wt, while SIRT1 overexpressing cells express less IGF-1 receptor than wt cells. STS treatment did not substantially alter such differences. In differentiated cells (Fig. 5B), IGF-1R expression was comparable among wt, SIRT1 overexpressing and SIRT1 silenced control cells. STS treatment induced an increase in IGF-1R expression in SIRT1 silenced cells. On the contrary, IGF-1R expression decreased after STS treatment in wt cells and more clearly in SIRT1 overexpressing cells (Fig. 5B).
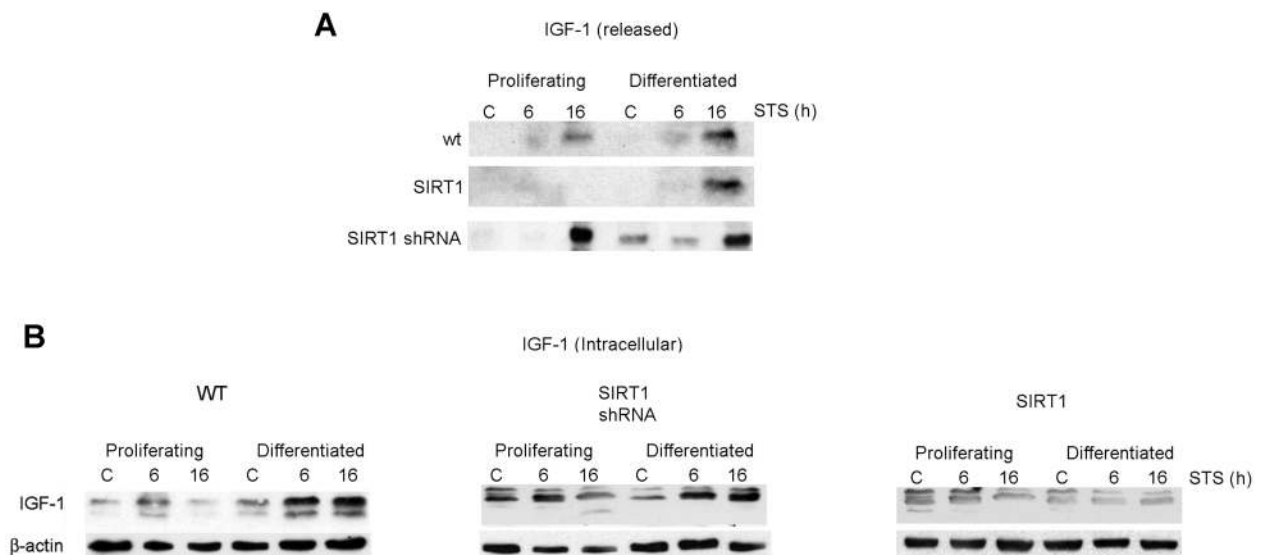

Fig. 4. IGF-1 expression and secretion in SIRT1 overexpressing and silenced cells. A: Proliferating and NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were either left untreated or treated with STS for the indicated times. Secreted IGF-1 levels were measured by Western blot after cellular culture medium collection and concentration as indicated in Material and Methods Section. C = control untreated cells. B: Proliferating and NaB differentiated wt, SIRT1 silenced, and SIRT1 overexpressing cells were either left untreated or treated with STS for the times indicated. Intracellular IGF-1 expression levels were measured by Western blot. β-actin was used as loading control. Results are representative of three independent experiments.
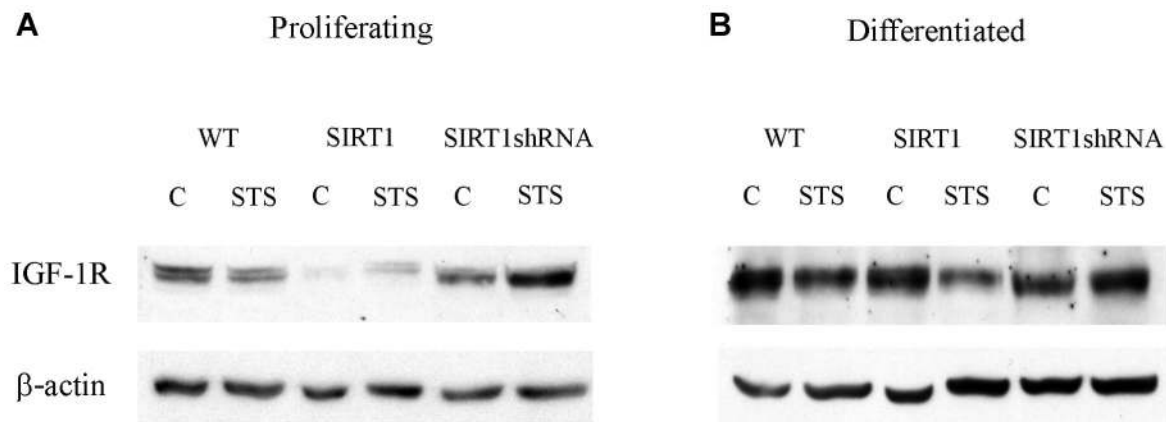
Fig. 5. IGF-1R expression in wt, SIRT1 overexpressing, and SIRT1 silenced cells. A: Proliferating wt, SIRT1 overexpressing and SIRT1 silenced cells were either left untreated or treated with STS for 6 h. IGF-1R levels were measured by Western blot as indicated in Materials and Methods Section. C = control untreated cells. B: NaB differentiated wt, SIRT1 overexpressing, and SIRT1 silenced cells were either left untreated or treated with STS for 6 h. IGF-1R levels were measured by Western blot as indicated in Materials and Methods Section. C = control untreated cells. Results in each part are representative of three independent experiments.

Increased AKT and ERK1/2 phosphorylation in SIRT1 silenced cells

Since IGF-1 and IGF-1R are more expressed in differentiated SIRT1 silenced NG108-15 cells compared to wt or SIRT1 overexpressing cells, we sought to determine if also the activation of downstream AKT and ERK1/2 kinases was increased in SIRT1 silenced cells. Therefore, wt, SIRT1 overexpressing and SIRT1 silenced cells were differentiated and then either left untreated or treated with STS. AKT and ERK1/2 activation was measured by determining phosphorylation levels. Figure 6A shows an increased phosphorylation of AKT on Tyrosine 308 in SIRT1 silenced cells after 30 min of STS treatment compared with wt cells. By contrast, no increase in AKT phosphorylation was observed in SIRT1 overexpressing cells (Fig. 6A). Total AKT levels were not affected by both SIRT1 overexpression or silencing and by STS addition (Fig. 6A). Similarly, ERK1/2 phosphorylation on Threonine 202/Tyrosine 204 was increased in wt, SIRT1 overexpressing cells and in SIRT1 silenced cells. The increased phosphorylation was more evident in SIRT1 silenced cells after 60 and 120 min of STS treatment (Fig. 6A). It is worth noting that, also total ERK1/2 levels were increased in SIRT1 silenced cells after STS treatment compared to wt and SIRT1 overexpressing cells. Densitometry analysis of AKT phosphorylation in wt, SIRT1 overexpressing and SIRT1 silenced cells is shown in Figure 6B. Figure 6C,D shows densitometric analysis of ERK1/2 phosphorylation on Thr202 and in Tyr204, respectively.

Discussion

This study showed that downregulation of SIRT1 protein expression increased neuronal resistance to cell death by activating IGF-1 signaling and consequently AKT and ERK pro-survival pathways. We demonstrated that neuronal secreted IGF-1 levels are influenced by SIRT1 protein levels in in vitro differentiated neurons. Our results indicated an IGF-1 neuroprotective role and a SIRT1 neurotoxic effect.

Initially, we investigated SIRT1 role in differentiated neurons observing the effect of sirtinol, a SIRT1 inhibitor. Sirtinol was indeed highly toxic in proliferating neuroblasts. Sirtinol toxicity was dramatically reduced in differentiated cells (Fig. 1A). SIRT1

expression in in vitro terminally differentiated neurons was measured. Our results showed that SIRT1 expression decreased in differentiated neurons, compared to their proliferating counterpart (Fig. 1B). A decrease of SIRT1 expression in terminally differentiated cells has been observed previously in a muscle differentiation model (Fulco et al., 2003) and in adipogenesis (Picard et al., 2004). From our previous studies we knew that differentiated neuronal cells are more resistant to STS treatment than their proliferating counterpart (Pucci et al., 2008). To understand if SIRT1 decrease in differentiated neurons could be responsible of such acquired cell resistance to STS, we produced SIRT1-silenced and overexpressing clones (Figs. 1C and S1). Silencing and overexpressing SIRT1 did influence p53 acetylation state. A decrease of p53 acetylation was observed in proliferating and differentiated SIRT1 overexpressing cells. On the contrary an increase of p53 acetylation was observed when SIRT1 expression was silenced (Fig. 1E). Interestingly, SIRT1 silencing had a positive effect on neuronal differentiation promoting neuronal net formation (Fig. 2A) and the increase of MAP5 (Fig. 2B). On the contrary, SIRT1 overexpression inhibited neuronal differentiation (Fig. 2A,B). Importantly, SIRT1 overexpression increased neurons sensitivity to STS, thapsigargin, camptothecin, and sorbitol (Figs. 2C and 3). SIRT1 has been indicated as neurotoxic also in other studies. SIRT1 overexpression in mice induced a memory deficit, and had no neuroprotective effects against damage induced by ischemia or by MPTP (Kakefuda et al., 2009). Moreover, Li et al. (2008) showed that SIRT1 inhibition protects neurons from oxidative stress. Experiments performed using SIRT1 chemical inhibitors highlighted that inactivation of SIRT1 was beneficial for neurons (Fulco et al., 2003). In particular, nicotinamide promoted neuronal survival after acute anoxic injury and fluid percussion injury (Chong et al., 2005; Holland et al., 2008). Finally, it has been shown that nicotinamide and sirtinol increases the resistance to glutamate and N-methyl-D-aspartate (NMDA)-induced excitotoxicity (Liu et al., 2008, 2009).

Our work showed that in differentiated SIRT1 overexpressing cells, the decrease of G0/G1 cells was associated to an increase of S phase cells indicating a positive effect of SIRT1 on proliferation and cell cycling. Indeed, SIRT1 overexpressing cells showed a decreased ability to differentiate
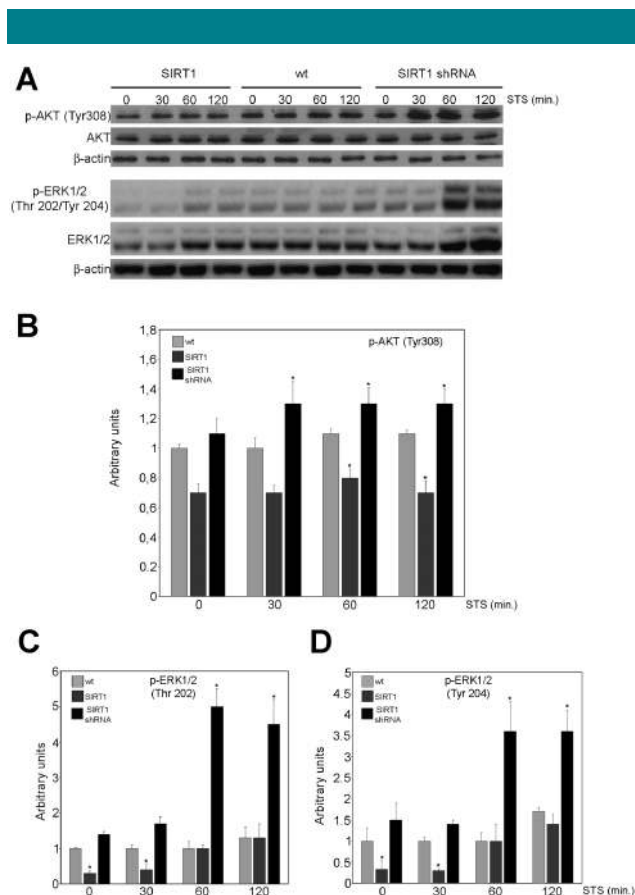
Fig. 6. AKT and ERK1/2 phosphorylation in wt, SIRT1 overexpressing, and silenced cells. A: NaB differentiated wt, SIRT1 overexpressing and SIRT1 silenced cells were either left untreated or treated with STS for the indicated times. AKT, pAKT, ERK1/2, and pERK1/2 levels were measured by Western blot as indicated in Materials and Methods Section. β-actin was used as loading control. B: Densitometric analysis of pAKT (Tyr308) Western blot after AKT and β-actin normalization. *, P < 0.05. C: Densitometric analysis of pERK1/2 (Thr202) Western blot after ERK1/2 and β-actin normalization. *, P < 0.05. D: Densitometric analysis of pERK1/2 (Tyr204) Western blot after ERK1/2 and β-actin normalization. *, P < 0.05. Error bars represent standard deviation (SD). Results in each part are representative of three independent experiments.

once treated with NaB (Fig. 2A central part). In SIRT1 proliferating cells the decrease in the percentage of G0/G1 cells was probably due to an increase in cell death associated to SIRT1 overexpression (Fig. 2C). The positive effect of SIRT1 on cell cycle has been also observed by Rathbone et al. (2009).

Since our previous work indicated that IGF-1 signaling positively influences cell survival in STS-treated neurons (Pucci et al., 2008), we investigated the correlation among SIRT1 expression and IGF-1 regulation in neuronal survival. We showed that, once treated with STS, IGF-1 expression and secretion increased in differentiated neurons compared to proliferating cells (Fig. 4). Such increase was more evident in SIRT1 silenced cells that expressed more IGF-1 even in proliferating conditions (Fig. 4).

Also IGF-1R expression levels were altered by SIRT1 expression manipulation (Fig. 5). In proliferating cells overexpressing or silencing SIRT1 induced respectively a decrease or an increase of IGF-1R expression in presence and in absence of STS treatment. In particular in SIRT1 silenced cells STS treatment induced an increase of IGF-1R (Fig. 5).

Considering our results on IGF-1 and IGF-1R, we tested the hypothesis that SIRT1 could influence IGF-1 pathway activation. As expected, AKT phosphorylation was strongly increased in SIRT1 silenced cells respect to wt cells (Fig. 6A,B). By contrast, in SIRT1 overexpressing cells AKT phosphorylation decreased during the treatment. It can be concluded that SIRT1 turns off IGF-1 activated AKT pathway. Indeed, AKT pathway resulted strongly activated in absence of SIRT1. ERK1/2 is also activated by IGF-1 (Laviola et al., 2007). In our system ERK1/2 phosphorylation increased in wt cells during STS treatment (Fig. 6A,C). The activation of ERK1/2 pathway, measured as Thr202/Tyr204 phosphorylation, was strongly augmented when SIRT1 expression was silenced (Fig. 6A,C). On the other hand, Thr202/Tyr204 phosphorylation on ERK1/2 was inhibited in SIRT1 overexpressing cells (Fig. 6A,C). These results confirmed that SIRT1 silencing in in vitro differentiated neurons enables the activation of AKT and ERK1/2 pathways.

Several published studies have shown that IGF-1 has a neurotrophic and neuroprotective role in central nervous system (Taguchi et al., 2007; Pucci et al., 2008; Broughton and Partridge, 2009). Our work indicated that downregulation of SIRT1 expression conferred resistance to STS induced cell death by augmenting IGF-1 survival pathways. Also Li et al. (2008) showed that SIRT1 inhibition can confer resistance to a cellular stress. In particular, they showed that SIRT1 inhibition induces resistance to neurons in presence of oxidative stress. However, in their system SIRT1 inhibition attenuates ERK1/2 activation by reducing IGF-1 signaling. On the contrary, here we observed that SIRT1 inhibition induced IGF-1 signaling. The contrasting data on IGF-1 pathway activation are probably explained by the fact that STS induces mostly apoptosis. Differently, oxidative stress induces mostly necrosis. Therefore, IGF-1 signaling can be differently regulated during necrosis or apoptosis. Indeed, the study by Li et al. (2008) did not highlight AKT activation as our results instead did.

Our study shows that in neurons the reparative role of IGF-1 is profoundly ameliorated in the absence of SIRT1 expression. Interestingly, SIRT1 silenced cells maintained IGF-1 secretion and IGF-1 receptor expression even in the presence of a cell death stimuli (STS). Such observation suggests that SIRT1 silencing by molecular or chemical strategies could increase the survival rate of damaged neurons though the IGF-1 pathway. Therefore, our work sets the foundations for mechanistic and pharmacological studies that, in a not so distant future, will help to understand if inhibition of SIRT1 expression and/or activity by new compounds can be used to reduce neuronal loss characteristic of neurodegenerative diseases.

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