

*Full Length Research Paper*

# Mutant huntingtin induced transfected PC12 cell death by overload of cholesterol

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**The present study was to explore whether overload of cholesterol elicited by Mutant huntingtin (mhtt) may directly induce transfected PC12 cell death. PC12 cells were transfected with huntingtin flanked with normal range of poly-Q (26Q) or mhtt (82Q). Expression of mhtt was turned on by addition of doxycycline (Dox). PC12 + Dox, Q26 + Dox and Q82 + Dox were continuously cultured for 60 days to monitor cell survival rate. The amount of cholesterol induced by mhtt was quantitatively analyzed by fluorescent intensities from filipin staining under confocal microscopy. Several key protein levels of huntingtin, caveolin1, and sterol regulatory element-binding proteins (SREBP-2) were determined by western blotting. Our results indicated that PC12 cells with Q82 were induced death around 25 days. Total numbers of survival cells were decreased to 40-50% by the end of 60 days in comparison with PC12 parental cells and Q26 cells. Cholesterol within Q82 cells started to accumulate from Day 18, with cholesterol overloaded around plasma membrane on Day 39, and eventually plasma membrane was collapsed and induced Q82 cells death on Day 60. Huntingtin and caveolin1 proteins in Q82 cells were greatly increased on 60 days; SREBP2 in Q82 cells was significantly cleaved and activated. Our results suggest that mhtt leads to cholesterol accumulation by over activated SREBP2, which stimulates cholesterol synthesis. The overload of cholesterol causes plasma membrane fragility and eventually induces transfected PC12 cell death.**

**Keywords:** Huntingtin (htt), mutant huntingtin (mhtt), SREBP2, PC12, cholesterol, cell death.

## INTRODUCTION

Huntington's Disease (HD) is defined as a progressive, autosomal dominant, neurodegenerative disorder characterized with a hall-mark of excessive CAG repeats encoding polyglutamine tract (polyQ>35 repeats) at the N-terminal fragment of mutant Huntingtin protein (Mhtt, exon 1 of HD gene) (Morell, V., 1993; Smith et al., 1993). This genetic defect results in dramatic loss of inhibitory gamma aminobutyric acid-ergic neurons in the caudate and putamen nuclei of striatum, as is shown in clinical symptoms—the jerky movements usually in chorea, and slow or limited motor movements, which become gradually irreversible (Quarrell et al., 2009).

Normal Huntingtin (htt) is found across various mam-

malian cells, and is required for normal embryonic development as neurogenesis. However, mhtt is cytotoxic whereas full length htt is neuroprotective. Truncated fragments of mhtt are much more cytotoxic than the full length of mhtt, and only N-terminal fragments but not C-terminal fragments are lethal and induce neuron apoptosis (Wellington et al., 2000; Lunkes et al., 2002). So far, several proteinases including the caspase family, calpains and apopain (CPP32) are found to have the capability of cleaving the N-terminal fragments of mhtt. The longer the expanded CAG triplet repeats in length, the more instable the polyQ tract, the more cytotoxic N-terminal fragments of polyQ generate in somatic and germline cells (Kim, Y.J., 2001). Discrete polyQ fragments of mhtt are more likely to aggregate than full-length fragments of mhtt. These aggregates cannot be sequestered out of the cytoplasm or degraded, and thus

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interfere with intracellular trafficking mediated by vesicular transporter—Caveolin (Trushina et al., 2006).

The central nervous system (CNS) is the most cholesterol-rich organ in all mammalian animals, and represents approximately 23% amount of total body cholesterol. Most brain cholesterol is unesterified and primarily accumulated in the plasma membranes of neurons and astrocytes of the CNS, whereas a paucity of cholesterol is found in endoplasmic reticulum (ER) and Golgi apparatus (Valenza and Cattaneo, 2006). Most cholesterol is endogenously synthesized, playing an important role in regulating membrane fluidity, permeability and serving as lipid rafts (Helms and Zurzolo, 2004). Activated sterol regulatory element binding protein-2 (SREBP-2) is responsible for regulating cholesterol synthesis and maintaining cholesterol homeostasis (Shimano, 2001). Alterations of several genes including lipid metabolism, vesicle trafficking and cell signaling at early transcription levels were found in mhtt knock-in transfected striatal ST14 cells (Zhang et al., 2006). Accumulation of cholesterol was previously found both in vitro-PC12 transfected cell line and in vivo-transgenic mice in our Lab (Trushina et al., 2006) (Toro et al., 2010).

Emerging evidence suggests that the cleavage of N-terminal fragments of mhtt may share the same protease with the precursor of SREBP-2. CPP32 (apoptain, caspase 3), a member of the family of cysteine proteases, is a prototypical caspase that has been activated during apoptosis (Wang et al., 1996). All of SREBP1 and 2, htt and Mhtt proteins have been found as substrates for CPP32. At least calpain1, 2, 5,7 and 10 have been implicated in htt and mhtt proteolysis, whereas caspase 1, 3, 8 and 9 are involved in htt cleavage. In transgenic mice, only caspase 6 but not caspase 3 is required for neuronal dysfunction and degeneration induced by mhtt. Moreover, sequential proteolysis of htt and mhtt by caspase and calpain may be taken place in different cellular compartments (Kim et al., 2001; Goldberg et al., 1996; Lunkes et al., 2002; Wellington et al., 2000; Graham et al., 2006; Maglione et al., 2006).

SREBP 1 and 2 are found to be cleaved and activated by caspase3, whereas CASP-2 and 7 genes that encode procaspase-2 and 7 are positively controlled by SREBPs. During cell apoptosis induced by cytokines, caspase-1 promotes cell survival by activating SREBPs (Wang et al., 1996; Sakai et al., 1996; Woo et al., 1997).

The primary goal of the present study was to gain insight into whether SREBP2 played a crucial role in excessive cholesterol accumulation induced by mhtt in transfected PC12 cells, and whether caveolin-1 mediated vesicle trafficking was altered or not. Our data indicated that SREBP2 and mhtt were cleaved concurrently, and superabundant cholesterol directly led to transfected PC12 death.

## MATERIALS

Filipin (from *Streptomyces filipinensis*) was purchased from Polysciences, Inc (Washington, PA). Caveolin 1 antibody was obtained from BD Biosciences. SREBP2, actin, HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Mouse anti-huntingtin protein monoclonal antibody (MAB2168) was purchased from Chemicon International, Inc. PC12 Tet-on system was purchased from Clontech (BD Biosciences). Doxycycline was purchased from Clontech (9BD Bioscience). Cell culture medium, serum, penicillin, streptomycin and polyornithine were purchased from Sigma.

## METHODS

### Transfected PC 12 cell culture

Tet-On system use a chimeric transactivator to regulate transcription of the mhtt gene from a silent promoter. The transactivator- Tet-regulated transcriptional activator (rtTA) is driven by the constitutive CMV promoter. In the presence of doxycycline, rtTA binds to the TRE and turns on the transcription of mhtt gene. Transfection of Tet-rtTA in full length huntingtin flanking with CAG trinucleotide repeats either 26 (normal) or 82 (pathological condition) into PC12 cells was constructed in our Lab (Trushina et al., 2006). Individual colonies were screened and sequenced for background and onset of mhtt gene expression in the presence of doxycycline (Dox). Parental PC-12 cells, tet-on Q82 or Q26 cells were

### Determination of cell survival rate over time

Briefly, parental PC12 cells, Q82 and Q26 transfected cells commenced to test within three generations. These tested cell lines grew to be confluent in RPMI 1640 medium with 2 mM glutamine, 5% heat inactivated fetal bovine serum and 5% heat inactivated horse serum. Cells were dispersed in flasks at cell concentration of  $0.5 \times 10^6$  cells/ml and maintained at 10%CO<sub>2</sub>/37°C/100% humidity. Experimental groups of PC12 cells + Dox (1µg/ml), Q82 + Dox (1µg/ml) and Q26 + Dox (1µg/ml) were performed in parallel. Six ml of  $0.5 \times 10^6$  cells/ml ( $3 \times 10^6$  total cells) was planted in a 75 cm<sup>2</sup> flask. Each treatment group was performed in quadruplicate by using separate 75 cm<sup>2</sup> tissue culture flasks. When cells grew to be confluent (every 3 days), cells were harvested, dispersed and passed down to another generation at the cell concentration of  $0.5 \times 10^6$  cells/ml. The remained cells were harvested and saved for western blotting. The total number of living cells in PC12+Dox were counted and

compared to those in parental PC 12 cells without Dox, whereas the total numbers of living cells in Q26+Dox, and Q82+Dox were compared to those in PC12+Dox, respectively, at certain time points (D5, D11, D18, D25, D32, D39, D46, D53 and D60). So the potential effect of doxycycline on cholesterol metabolism in PC 12 and derivative cell lines were excluded. When counting cell numbers, the cell suspension was titrated into sequential orders as 1:2, 1:4 and 1:8, and cell numbers in each suspension were calculated from at least 5 random selected microscopic fields. The total numbers of different treatment cells were calculated by multiplying dilution factors. Living cells were identified by trypan blue exclusion under a microscope, where dead cells with trypan with blue inclusion were excluded from the total cell number. Two independent experiments were conducted sequentially.

### Filipin staining

Fluorescent filipin staining was adapted from previous method (Klinkner et al., 1997; Castanho and Prieto, 1992). In parallel with the above experiment, three tested cell lines of PC12+Dox, Q26+Dox and Q82+Dox were cultured on cover slips in 6-well plates (pre-incubated with 0.1% polyornithine medium overnight) and terminated at pre-determined time point of culture (Day0, Day5, Day18, Day39 and Day60). After removing the medium, cover slips were gently washed with cold PBS one time, and incubated with 2%glutaraldehyde for 15 min at room temperature. Cover slips were gently washed with cold PBS three more times, and then blocked with 3% bovine serum albumin (BSA) for 30 min, permeated with 0.2% Triton X-100 in PBS for 10 min at room temperature, and finally cover slips were incubated with filipin solution (100 µg/ml in methanol) for 30 min. The cover slips were completely dried and mounted on the slides with prolong gold anti-fade reagent. The images were captured by a Zeiss 510 confocal microscope. The integrated blue intensity (filipin specifically binds to cholesterol) were calculated from randomly selected 100 cells and quantitatively analyzed with Meta Morph Premier Software (7.5 version).

### Living cell imaging

Three tested cell lines of PC12+Dox, Q26+Dox and Q82+Dox were cultured in 35 mm petri dishes embedded with cover slips (pre-coated with poly-d-lysine, MatTek) at the bottom. In parallel with the above experiment, 57 day aged cells (continuously cultured for 57 days after adding Dox) were planted at  $0.5 \times 10^6$  cells/ml, and until Day 60 living cell images were captured in phase contrast under a Olympus microscope magnified at 10 times.

### Western blotting

A standard western blot procedure was performed according to our previous study (Roy and McMurray, 2001; Xu and Rajanna, 2006; Xu, 2007; Xu, 2010). Briefly, cells were lysed with RIPA buffer (150 mM sodium chloride, 1%Triton X-100, 0.5%sodium deoxycholate, 0.1%sodium dodecyl sulfate (SDS), 50 mM Tris (pH8.0)) with 2 mM PMSF and proteinase inhibitor cocktail. The suspension of cell lysates was sonicated, and centrifuged at 15,000 x g for 10 min at 4 °C. The milky suspension was collected as measuring samples and cell debris were discarded. The sample was adjusted to 10% glycerol, and equal amount mixed with 2 X loading buffer (the final concentration as 58.3 mM Tris HCl pH6.8, 1.6% SDS, 5%glycerol, 2.5% 2-mercaptoethanol, 0.002%bromophenol blue), snap frozen in liquid nitrogen and stored at -20 °C. Protein concentration was determined using Lowry's method (Lowey et al., 1951). To determine expression of mhtt, each sample at equal amount of protein without heat denaturation was loaded on a 10%acrylamide/0.05%bisacrylamide gel; meanwhile, to determine expressions of SREBP-2, caveolin-1 and loading control-actin, each sample at equal amount protein was heat-denatured and separated by 7.5% SDS-PAGE gel and transferred on to PVDF membrane. The membrane was probed with correspondent individual first antibodies, and conjugated with second antibodies, eventually developed with chemiluminescent medium and exposed on X-ray film. The bands on films were scanned by using Un-scan-it software from Silk Scientific, Inc (Orem, Utah). Independent experiment was repeated three times.

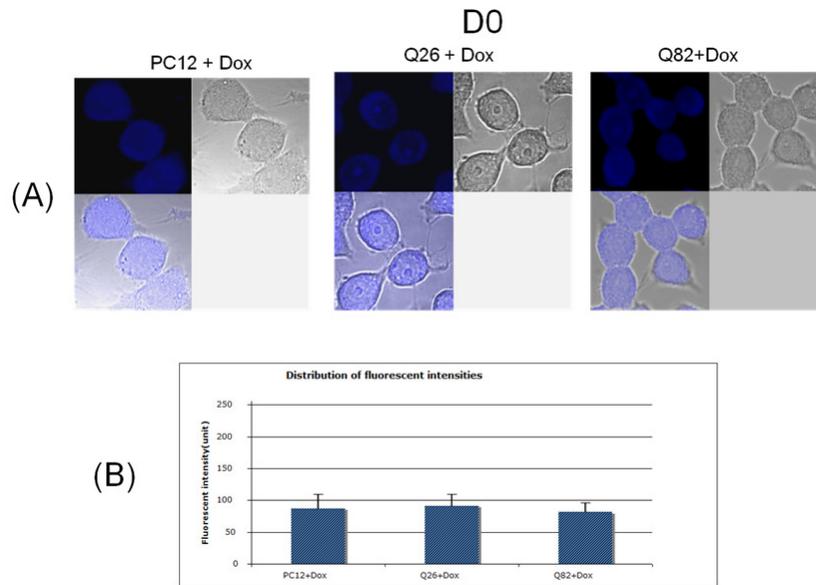
### Statistical Analysis

All the quantitative data were expressed as mean  $\pm$  SD, and subjected to statistical analysis by using One-way ANOVA program (SigmaStat 3.0).

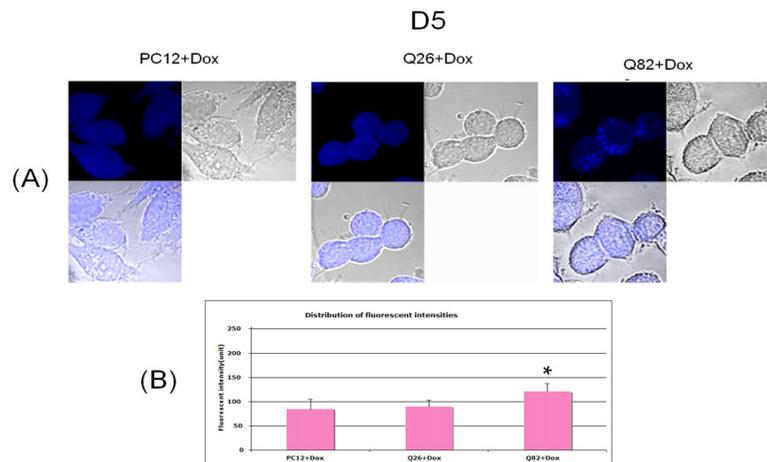
## RESULTS

### Cholesterol accumulation led to transfected PC12 cell apoptosis

Previous reports have demonstrated or implicated mhtt or its fragments to induce striatal neuron degeneration, or enhance NMDA receptor elicited cell death. However, how mhtt directly induced cell apoptosis remained unaddressed. Previous studies in our Lab indicated that mhtt induced cholesterol accumulation in vivo and in vitro, but transient expression of mhtt in short period of cell culture was found only inducing slight cytotoxicity



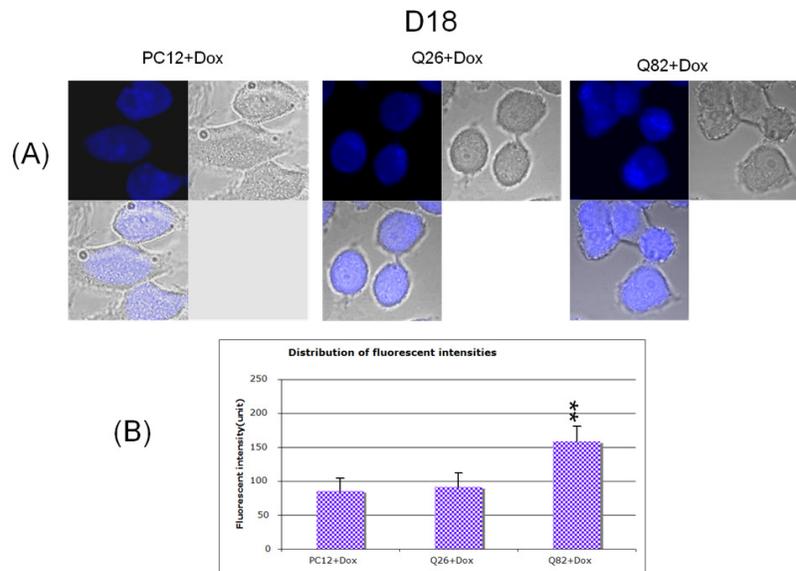
**Figure 1.** Fluorescent intensity analysis of cholesterol stained with filipin among PC12+Dox, Q26+Dox and Q82+Dox cells on Day0. (A): Images of filipin stained cells captured in blue, phase contrast and overlap channels under an LSM 510 confocal microscope. (B): Integrated fluorescent intensities from random selected 100 cells were determined with Meta Morph Premier Software (7.5 version) and analyzed in statistics with One-way ANOVA program (SigmaStat 3.0).



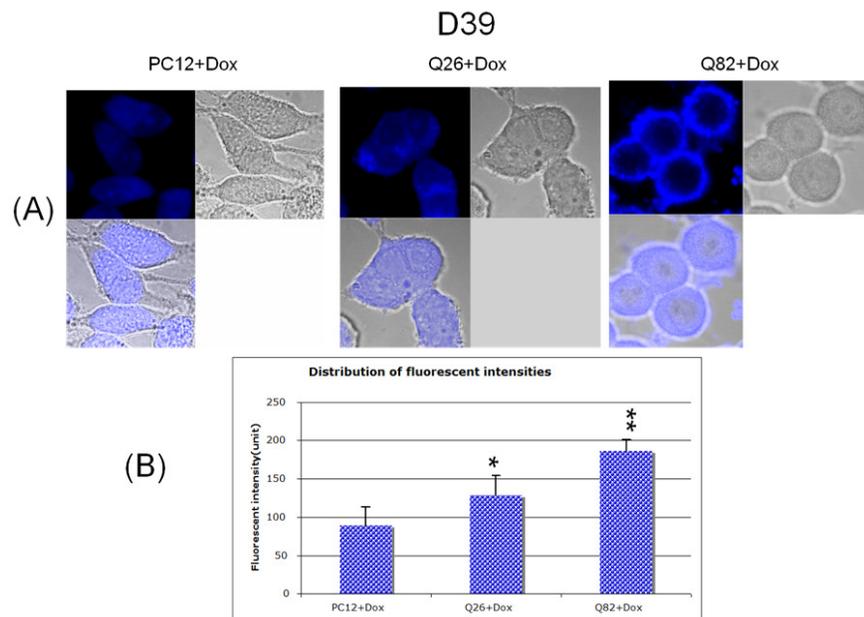
**Figure 2.** Fluorescent intensity analysis of cholesterol stained with filipin among PC12+Dox, Q26+Dox and Q82+Dox cells on Day5. (A): Images of filipin stained cells captured in blue, phase contrast and overlap channels under an LSM 510 confocal microscope.. (B): Integrated fluorescent intensities from random selected 100 cells were determined with Meta Morph Premier Software (7.5 version) and analyzed in statistics with One-way ANOVA program (SigmaStat 3.0).(\*P<0.05)

(Trushina et al., 2006). In the present study, we transfected mhtt into PC12 cell line and extended expression of mhtt for two month, and then observed the

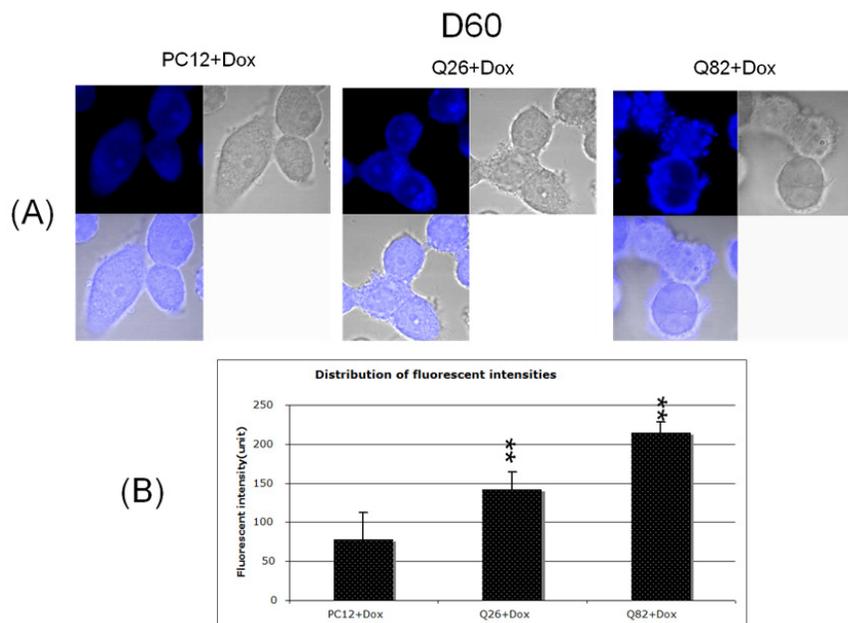
final consequence that overload of cholesterol induced cell death. As shown in Figure 1-5, on Day 0, cholesterol did not accumulated by filipin staining among parental



**Figure 3.** Fluorescent intensity analysis of cholesterol stained with filipin among PC12+Dox, Q26+Dox and Q82+Dox cells on Day18. (A): Images of filipin stained cells captured in blue, phase contrast and overlap channels under an LSM 510 confocal microscope.. (B): Integrated fluorescent intensities from random selected 100 cells were determined with Meta Morph Premier Software (7.5 version) and analyzed in statistics with One-way ANOVA program (SigmaStat 3.0). (\*\*P<0.01).



**Figure 4.** Fluorescent intensity analysis of cholesterol stained with filipin among PC12+Dox, Q26+Dox and Q82+Dox cells on Day39. (A): Images of filipin stained cells captured in blue, phase contrast and overlap channels under an LSM 510 confocal microscope.. (B): Integrated fluorescent intensities from random selected 100 cells were determined with Meta Morph Premier Software (7.5 version) and analyzed in statistics with One-way ANOVA program (SigmaStat 3.0).(\*P<0.05, \*\*P<0.01)



**Figure 5.** Fluorescent intensity analysis of cholesterol stained with filipin among PC12+Dox, Q26+Dox and Q82+Dox cells on Day60. (A): Images of filipin stained cells captured in blue, phase contrast and overlap channels under an LSM 510 confocal microscope. (B): Integrated fluorescent intensities from random selected 100 cells were determined with Meta Morph Premier Software (7.5 version) and analyzed in statistics with One-way ANOVA program (SigmaStat 3.0). (\*\* $P < 0.01$ )

PC12+Dox, Q26+Dox and Q82+Dox cells. On Day 5, highlighted blue spots scattered in Q82+Dox cells, which indicated cholesterol accumulation by filipin staining, whereas such accumulation were found neither in PC12+Dox nor Q26+Dox. On Day 18, shiny blue spots merged together and occupied bulk of cytoplasm room, whereas a few spots appeared in Q26+Dox cells but not in parental PC12+Dox cells. On Day 39, a large amount of cholesterol was overloaded and accumulated around outer cytoplasm membrane, while some cholesterol was accumulated in Q26+Dox cells but not in PC12+Dox cells. On Day 60, overloaded cholesterol led to cell plasma membrane collapsed, cell death or lysis, whereas merged shiny blue spots spread in Q26+Dox cells but not in PC12+Dox cells. Integrated fluorescent intensities averaged from randomly selected 100 cells were analyzed. On Day 5, total fluorescent intensity in Q82+Dox group was higher than in Q26+Dox and PC12+Dox groups ( $P < 0.05$ ). On Day 18, total fluorescent intensity in Q82+Dox group was greatly higher than in Q26+Dox and PC12+Dox groups ( $P < 0.01$ ). On Day 39, total fluorescent intensity in Q82+Dox group was dramatically higher than in PC12+Dox group ( $P < 0.01$ ), and total fluorescent intensity in Q26+Dox group was also higher than in PC12+Dox group ( $P < 0.05$ ). On Day 60, total fluorescent intensity in Q82+Dox group was significantly higher than in PC12+Dox group ( $P < 0.01$ ), and total fluorescent intensity in Q26+Dox group was also

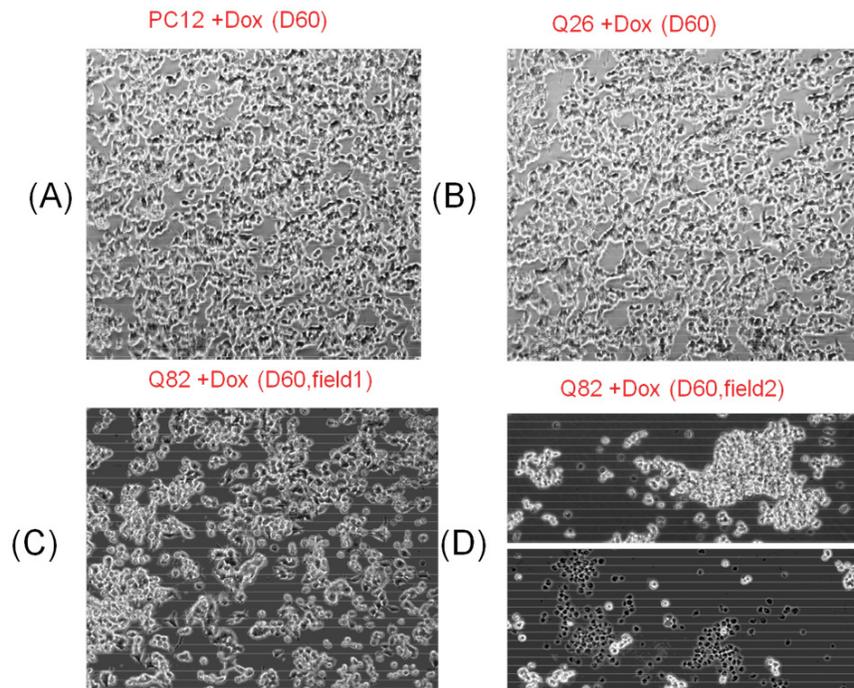
much higher than in PC12+Dox group ( $P < 0.01$ ). As indicated on Day 60, Q82+Dox cells (PC12 cells transfected with Q82 in the presence of Dox) underwent lysis.

#### Microscopic depiction of transfected PC12 cell death

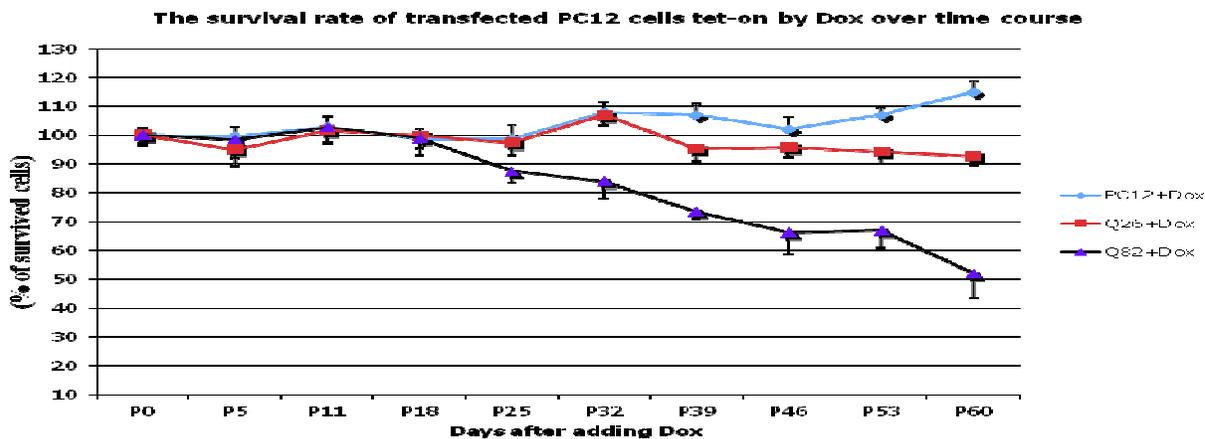
Concurrently, parental PC12+Dox, Q26+Dox and Q82+Dox cells were observed under phase contrast microscopy at low magnification (10 times). As shown in Figure 6, on Day 60, Q82+Dox cells detached from the dishes, clustered and floated, which indicated cells were dead; some only left debris. In comparison with Q82+Dox cells, parental PC12+Dox and Q26+Dox cells were evenly spread on the dishes and shiny. These three group cells were also harvested and characterized with trypan blue staining. The results indicated that most cells in Q82+Dox group were dead, but more than 95% cells were alive in both Q26+Dox group and parental PC12+Dox group (data not shown here).

#### Dynamic delineation of transfected PC12 cell survival rate

Since mhtt inducing neuron degeneration is a long-term process, it raises a question how soon mhtt and frag-



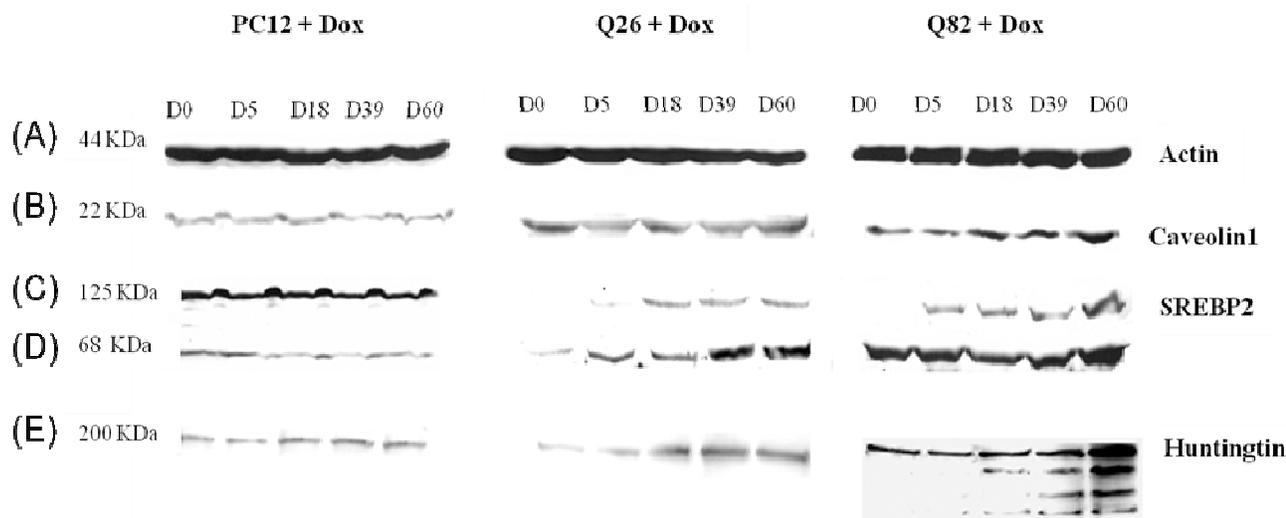
**Figure 6.** Images of PC12+Dox, Q26+Dox and Q82+Dox cells cultured on Day60 after addition of Dox are observed under a phase contrast microscope (x10 magnification). (A): PC12+Dox (Day60). (B): Q26+Dox (Day60). (C): Q82+Dox(Day60, field1). (D): Q82+Dox(Day60, field2).



**Figure 7.** Survival rates of PC12+Dox, Q26+Dox and Q82+Dox cells after addition of Dox over time course. Total number of living cells in PC12+Dox were counted and compared to those in parental PC 12 cells without Dox, whereas total numbers of living cells in Q26+Dox, and Q82+Dox were compared to those in PC12+Dox, respectively, at certain time points (D5, D11, D18, D25, D32, D39, D46, D53 and D60). Statistical analysis was applied with One-way ANOVA program (SigmaStat 3.0).(\*P<0.05, \*\*P<0.01)

ments can cause neurons death. The parental PC12 cell lines transfected with normal range of poly-Q repeats (26Q) and pathological range of poly-Q repeats (82Q), respectively, are good models to explore the underlying

mechanism following chronic cytotoxicity induced by mhtt. As shown in Figure 7, after continuous culture for 60 days, PC 12 cells in the presence of Dox were comparable to PC 12 cells without Dox. On Day 25, the



**Figure 8.** Protein expression profiles of actin, caveolin1, SREBP2 and Huntingtin extracted from PC12+Dox, Q26+Dox and Q82+Dox cells after addition of Dox over Day 0, 5, 18, 39 and 60. (A): Actin. (B): Caveolin. (C): SREBP2. (D): Huntingtin.

survival rate of Q82+Dox cells decreased significantly to  $87.6 \pm 4.1\%$  in comparison with that of PC12+Dox cells ( $P < 0.05$ ), which indicates Q82+Dox cells commenced death. On Day 32, the survival rate of Q82+Dox cells was greatly decreased to  $84 \pm 6.0\%$  against that of PC12+Dox cells ( $P < 0.01$ ). On Day 39, the survival rate of Q82+Dox cells was dramatically decreased to  $73.5 \pm 2.5\%$  against that of PC12+Dox cells ( $P < 0.01$ ), and the survival rate of Q26+Dox cells was slightly decreased to  $95.3 \pm 4.4\%$  against that of PC12+Dox cells ( $P < 0.05$ ). From Day 46, 53 to Day 60, survival rates of Q82+Dox cells were tremendously decreased to  $66.3 \pm 7.5\%$ ,  $67 \pm 6.1\%$  and  $52 \pm 8.5\%$ , respectively, against those of PC12+Dox cells ( $P < 0.01$  in all). Nevertheless, on Day 53 and 60, survival rates of Q26+Dox cells were significantly decreased against those of PC12+Dox cells in statistics ( $P < 0.01$ ), actual losses of Q26+Dox cells were found within 10%.

### Proteolysis crosslink between Mhtt and SREBP2

Full length mhtt was deemed to take sequential cleavages or proteolysis. Truncated mhtt fragments were more readily to aggregate than pro-full length mhtt. Aggregates of mhtt forming nuclear inclusions (NI) were thought to cause cellular dysfunction in polyQ disorders, since NIs were detectable prior to onset of symptoms in HD mouse models and human patient brains. Herein our data revealed another mechanism that overabundant cholesterol leads to Q82+Dox cells death. However, it raises questions where cholesterol comes from. SREBP2 is a key transcription factor that is responsible for

cholesterol synthesis or uptake. SREBP2 must be activated via cleavage and translocated into nucleus to serve its role. Attempt to link cleavages between mhtt and SREBP2 is a key in our story.

However, before there are two issues needed to be classified. First, in terms of Day0, Day 0 was assigned as the origin of this study. In fact, selected cell lines—Q26 and Q82 were taken out from the storage in liquid nitrogen and placed to culture for at least three generations in order to ensure its stability. Insertion of outer genes should disturb regular expression of other genes. Therefore, protein expressions of SREBP2 in Q26 and Q82 cells were not identical with that in PC12 cell. Second, insertion of Q26 was noted to increase SREBP2 cleavage and a little increase of mhtt, and slight cholesterol accumulation even 26 CAG repeats were fallen into normal range. The mechanism was barely unknown. As shown in Figure 8, on Day 18, mhtt had been cleaved into four traced bands. On Day 39 and 60, the more mhtt protein found, the more cleaved fragments appeared, but cleaved sites seemed to stay at the same amino sequence, because only four bands were found and became heavier and darker from Day 18 to Day 39, and 60. Meanwhile, a little endogenous huntingtin was constantly expressed in PC 12+Dox cells from Day 0 to Day 60, but slight mhtt protein was slightly increased from Day 18 to Day 60, but no cleaved fragments were found. Interestingly, a similar pattern of SREBP2 cleavage was found in Q82+Dox cells in comparison with mhtt cleavage in Q82+Dox cells. The differences were: 1) SREBP2 was only cleaved into two bands from 125 KDa (precursor) to 70 KDa (SREBP2). 2) Since precursor of

SREBP2 was converted to activated form- SREBP2, so the less the precursor showed on the upper band, the more activated SREBP2 appeared on the lower band. 3) The precursors of SREBP2 from Day 0 to Day 60 were gradually decreased, while SREBP2 activated forms were proportionally increased in Q82+Dox cells. In addition, there were slight increases of SREBP2 in Q26+Dox cells from Day 5 to Day 60; however, precursors and SREBP2 in PC 12+Dox cells were kept constant from Day 0 to Day 60. As regarding to caveolin1, a slight increase was found in Q82+Dox cells on Day 60 compared to expressions of caveolin1 in PC 12+Dox and Q26+Dox cells.

## DISCUSSION

The current results reveal a novel mechanism that overabundant cholesterol directly induces death of transfected PC12 cells with 82 expanded polyQ tracts in mutant Huntingtin (mhtt) in vitro. Overabundant cholesterol is due to overactivation of SREBP2, whose cleavage is paralleled with the cleavage of mhtt. The linkage between SREBP2 activation and mhtt cleavage may unveil a novel mechanism of striatal neuron loss in Huntington's disease.

Amount of cholesterol in CNS is delicately regulated by SREBP2. Most cellular cholesterol (about 85%) is unesterified and docking at plasma membrane. The ratio of cholesterol to phospholipids is important to maintain membrane fluidity and lipid rafts function. Only 5% cholesterol left at endoplasmic reticulum (ER), but plays an important role in regulating cellular cholesterol homeostasis (Valenza and Cattaneo, 2006; Soccio and Breslow, 2004). Cholesterol accumulation has been observed in many neurodegenerative diseases, e.g., Alzheimer's disease, and Niemann-Pick (NPC) disease (Schweitzer et al., 2009; Valenza and Cattaneo, 2006). Cholesterol accumulation was found in mhtt transgenic mice and transfected PC12 cells in our previous study (Trushina et al., 2006) (Toro et al., 2010).

The phenomenon leaves an open question: where does the cholesterol come from? Previous studies implicated that brain local cholesterol is mainly dependent on local *de novo* synthesis rather than from uptaking from LDL (Valenza and Cattaneo, 2006; Soccio and Breslow, 2004). In NPC mode brain, neuronal cholesterol accumulation precedes neuron degeneration and loss *in vivo* (Schweitzer et al., 2009). Cholesterol synthesis is due to activation of SREBP2. Under physiological conditions, when cells are depleted of cholesterol, SREBP2 is activated by two sequential proteolytic cleavages, and eventually it releases the N-terminal transcription factor from the membrane, thereby allowing the mature form to enter the nucleus and activate transcription target genes (hydroxyl-methylglutaryl (HMG)-CoA synthase and reductase—key

rate-controlling cholesterol biosynthetic enzymes). SREBP2 is first cleaved by Site-1-Protease (S1P), which is a rate-limiting step, and followed cleaved by the Site-2-Protease (S2P) (Soccio and Breslow, 2004; Shimano, 2001). However, SREBP2 was also able to be cleaved by CPP32 during death.

CPP32 (apopain, caspase 3), a cysteine protease, was shown previously to cleave SREBP2 in vitro at an aspartic acid between the basic helix-loop-helix leucine zipper domain and the first transmembrane domain, thereby liberating a transcriptional active form. Moreover, such a cleavage of SREBP2 was not inhibited by feedback of cholesterol. Previous studies indicated that CPP32 itself and SREBP2 were activated during apoptosis induced by staurosporine (Wang et al., 1996; Goldberg et al., 1996; Sakai et al., 1996). However, there is no direct evidence shown that calcium-dependent calpain family members are able to cleave SREBP2.

Meanwhile, mhtt is found to be an unspecific substrate for quite a few enzymes including caspase 3 (CPP32)/caspase 6, and calpain family (calpain 1, 5, 7 and 10), as these proteases have relaxed primary sequence specificities for the cleavage site of mhtt. An impairment of mhtt clearance results in aggregation of mhtt fragments (Goldberg et al., 1996; Maglione et al., 2006; Graham et al., 2006; Kim et al., 2001; Lunkes et al., 2002). Combining with previous findings in the literature, we can infer both SREBP2 activation and mhtt cleavage share a similar proteolytic process based on the following facts:

- 1) Both SREBP2 and Mhtt are apopain (CPP32) substrates;
- 2) Both SREBP2 activation and mhtt cleavage take sequential and multiple proteolysis;
- 3) SREBP2 and mhtt were found similar size cleaved products (activated and mature SREBP2—68 KDa, Mhtt fragments between 52 and 89 KDa);
- 4) Cleavage of Mhtt is associated with SREBP2 activation—cholesterol accumulation, which takes place prior to death.

Many pathological changes have been found in HD. Recently, nuclear inclusions of mhtt fragments have been received a great deal of attention. Formation of nuclear inclusions changes many genes regulation at transcriptional level. The addition of a nuclear export signal (NES) to mhtt helps to sequester and export mhtt from the nucleus; mhtt completely loses its ability to induce striatal neurodegeneration (Lunkes et al., 2002). The poly-Q expanded mhtt adapts itself to a novel conformation, which renders them prone to aggregate and conveys toxic effects. Either nuclear inclusions or cytoplasmic inclusions, therefore, become a pathologic hallmark of disorders in HD patients and transgenic mice model systems. In addition, the presence of expanded poly-Q tract increases the rate of cleavage of mhtt. The longer the poly-Q tract in length at mhtt is, the faster the cleavage rate at mhtt is, and the more neuronal toxicity

Mhtt elicits. However, neither nuclear inclusions nor cytoplasmic inclusions can directly lead to neuron death.

Our data demonstrated that overload cholesterol due to over activation of SREBP2 directly induces transfected PC12 cell death, which is accompanied by mhtt cleavage. In addition, mhtt and caveolin-1 functionally interact with each other and leads to cellular trafficking defects. Striking accumulation of mhtt fragments or cholesterol also slows down the recycle of caveolin-1, and causes slight accumulation of caveolin-1 (shown in our data).

In normal range of poly-Q knocked-in cell line-- Q26+Dox cells, slight cholesterol accumulation was found as well. The reason is due to: 1) knocked -in exogenous gene might interfere with cellular function, as PC12 cells proliferates fast. 2) Though Dox may affects cholesterol metabolism, all three cell lines were tested in the presence of Dox. Effect of Dox can be excluded. 3) On Day 53 and 60, that Q26+Dox cells were statistically decreased compared to PC12+Dox cells was due to the relative increase of PC12+Dox cells. In fact, the survival rate of Q26+Dox cells is close to 100% (92-95%).

Our findings open the possibility that intervene with onset of neuron degeneration in HD through inhibiting SREBP2 activation. A selective protease inhibitor for over activation of SREBP2 might present a new avenue to tackle the disorder of HD. However, a firm conclusion as to the link between SREBP2 activation and Mhtt cleavage awaits detailed studies in the future.

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