ャプチャーを行い、Illumina Hiseq2000 sequencer を用いたエクソーム解析を行った。

(倫理面への配慮)

研究はヒトゲノム遺伝子解析研究に関する倫理指針および臨床研究に関する倫理指針を遵守して実施した。ALS 患者コホートの構築、網羅的ゲノム解析については、参加するすべての施設で倫理委員会承認を得た。研究対象者には倫理委員会にて承認された説明書・同意書を用いて十分な説明を行い、文書同意を得て参加いただいた。検体・資料を分析する際には、氏名・住所・生年月日などの個人情報を取り除き、匿名符号をつけ、連結可能匿名化して厳重に管理した。

C.研究結果

合計の ALS508 例のうち、家族歴がなく、El Escorial 診断基準で possible 以上と診断され、ALS と関連があるとされる *C9ORF72* 遺伝子の繰り返し配列の延長が認められなかった、孤発性 ALS469 例について、28 種類の既知の ALS 関連遺伝子エクソンの網羅的シークエンスを解析した。その結果、28 遺伝子のエクソン領域内で、既に ALS 発症の原因と関連すると報告されている既知の遺伝子変異が 14 例で認められた。内訳は、SOD1 11 例、FUS 2 例、TARDBP 1 例であった。さらに、28 遺伝子のエクソン領域内で、dbSNP、HGVD などのデータベース上に存在しない、新規の variant について検討を行い、115 個の新規 variant を抽出した。さらに PolyPhen2 で possibly or probably damaging または SIFT で Damaging と判定された病原性の疑われる variant を抽出し、Sanger sequence 法で確認したところ、最終的に 30 例で計 35 個の variant を確認した。内訳は、*ALS2* 1 例、*ATXN2* 1 例、*C9ORF72* 1 例、*DAO* 1 例、*DCTN1* 5 例、*FIG4* 3 例、*PRPH* 2 例、*RNF19A* 3 例、*SETX* 4 例、*SPG11* 8 例、*TAF 15* 1 例、*TFG 1* 例、*ZNF512B* 4 例であった。

D.考察

今回の解析で、孤発性 ALS 患者の中に、14 例で既知の家族性 ALS 遺伝子変異を認めた。これらの変異に関しては、浸透率の低い病原性の遺伝子変異である可能性、あるいは De novo mutation が起こった可能性、あるいは上の世代での発症が見逃されている可能性などが考えられた。また、今回見出した新規の variant に関しては、単独で ALS を発症させる新規の遺伝子変異の可能性、あるいは、単独では ALS を発症させないが、ALS の発症に寄与する rare variant の可能性があると考えられた。

E.結論

孤発性 ALS 469 例に対して ALS 疾患関連 28 遺伝子のエクソン領域について網羅的な遺伝子解析を施行し、14 例に既知の家族性 ALS 遺伝子変異を認め、30 例に病原性の疑われる新規の variant を認めた。今後、分子生物学的な検討をすすめていく予定である。

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G.知的財産権の出願・登録状況
特記なし。

厚生労働科学研究委託費（難治性疾患実用化研究事業）
委託業務成果報告（業務項目）

孤発性 ALS 患者大規模前向きコホートの臨床バイオリソース・ゲノム遺伝子・不死化細胞を用いた病態解明、治療法開発研究

孤発性 ALS 患者由来 iPS 細胞ライブラリーの作製、病態探索、治療標的分子探索、薬剤シーズ探索

疾患特異的 iPS 細胞を用いた運動ニューロン病の病態解析

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研究要旨

筋萎縮性側索硬化症（ALS）などの運動ニューロン病の疾患特異的 iPS 細胞を用いた病態モデルを作成するために、ヒト多能性幹細胞から、遺伝子導入を行わずに迅速・高効率に運動ニューロンを誘導する培養法を確立した。また運動ニューロン特異的なレポーターレンチウイルスを作成し、生きたままで運動ニューロンを標識・可視化する手法を構築した。これらの技術を組み合わせて用いることで、疾患特異的 iPS 細胞を用いた運動ニューロン変性メカニズムの解明に応用可能であると考えられた。

A.研究目的

これまでの神経変性疾患の病態解析や治療法開発においては、主に動物モデルや培養細胞株などが用いられてきたが、これらのモデルは必ずしも患者の病態を忠実に再現し得ないこと、また、種間の差を避けられないことなどから、より患者の病態に即したヒト疾患モデルが求められてきた。近年開発された iPS 細胞の技術を用いることで、患者の体細胞をリプログラミングして多能性幹細胞を作成し、対象疾患において障害をうける神経系細胞（疾患感受性細胞）へと分化誘導することで、患者自身の神経系細胞を用いた病態解析や薬剤開発が可能になった。そこで、本研究では、筋萎縮性側索硬化症（ALS）、脊髄性筋萎縮症（SMA）、球脊髄性筋萎縮症（SBMA）など、運動ニューロンが選択的に障害をうける運動ニューロン病に焦点をあて、患者体細胞から iPS 細胞を樹立して運動ニューロンへと分化誘導することで新たな疾患モデルを作成し、病態解明や新規治療法の開発へ応用するための技術基盤を確立することを目的に行う。

B.研究方法

ヒト多能性幹細胞は、マイトマイシン処理したマウス胎児線維芽細胞、または SNL フィーダー細胞上でヒト ES 細胞培地（20% KSR、4ng/ml bFGF 含有）を用いて維持培養を行い、分化誘導に用いた。分化誘導開始後、経時的に RNA を採取し定量的 RT-PCR により遺伝子発現解析を行った。その際、各種阻害剤を添加して、分化誘導に与える影響を検討した。また、接着分化した細胞は、細胞種特異的マーカーの免疫染色を行い、細胞の特性解析を行った。

レンチウイルスは、レンチウイルスベクタープラスミドを、パッケージングプラスミドとともに 293T 細胞へトランスフェクションし、培養上清を回収することにより作成した。レポーター遺伝子の発現は、抗 GFP 抗体を用いた免疫染色による確

認を行った。

（倫理面への配慮）

本研究におけるヒト iPS 細胞を用いた研究に関しては、「臨床研究に関する倫理指針」および「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守し、愛知医科大学医学部倫理委員会において、「神経疾患患者からの iPS 細胞の樹立とそれを用いた疾患解析に関する研究」として承認を受けている。レンチウイルスを用いた実験は、「遺伝子組み換え生物等の使用等の規制による生物の多様性確保に関する法律」（カタルヘナ法）等を遵守し、愛知医科大学組換え DNA 実験安全委員会の承認を受けた上で実施した。

C.研究結果

1.ヒト iPS 細胞の運動ニューロンへの迅速・高効率分化誘導法の開発

これまでに、ヒト ES 細胞、ヒト iPS 細胞から胚様体（Embryoid body: EB）を介して、ニューロスフェアとして高効率に神経幹細胞を誘導する培養法を確立してきたが、この方法では、EB の形成に約一か月間、さらにニューロスフェアの誘導に 1-2 か月間と長期間の培養を要した。そこで、神経分化に対して抑制的に働く Nodal シグナル・BMP シグナルの下流で働く Smad シグナルを阻害する Dual Smad inhibition 、すなわち BMP 阻害剤である Dorsomorphine と TGFβ阻害剤である SB431542（Chambers *Nat Biotech* 2009）を用い、さらに神経分化に促進的に働くと考えられる GSK3β阻害剤（BIO）を分化誘導の初期に添加することで、神経分化誘導の効率化と培養期間の短縮を試みた。その結果、Dual Smad inhibition のみでも、従来よりも短期間の分化誘導で神経幹細胞のマーカーである *SOX1* の有意な発現上昇が得られ、さらに GSK3β阻害剤を添加することにより、従来通り培養した場合と同程度の *SOX1* の発現を得ることができた。またこの条件下で、高効率に神経幹細胞を誘導することに成功した（図 1 、Numasawa et al., *Stem Cell Reports* 2014）。

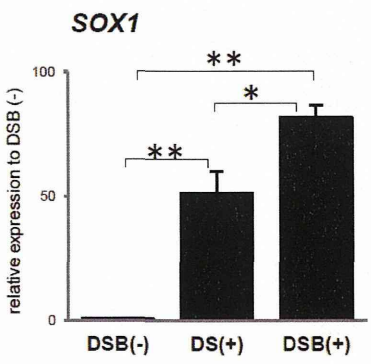

図 1　ヒト多能性幹細胞の神経分化における Dual Smad inhibition、GSK3β阻害剤の効果

次に、EB の培養中に運動ニューロンの発生に重要な後方化因子であるレチノイン酸（RA）と Sonic hedgehog （Shh）シグナルを活性化する Purmorphamine を添加して経時的な遺伝子発現変化を解析したところ、分化誘導開始後すぐに神経幹細胞のマーカーである *SOX1* や *PAX6*、および proneural gene である *NGN2* の発現上昇がみられ、その後速やかに、運動ニューロンの前駆細胞で発現する *OLIG2* の顕著な発現上昇が観察された。またこの時期には、オリゴデンドロサイト前駆細胞で発現する *NKX2.2* の発現上昇も観察された。そこで、この培養条件で培養した EB を接着培養で分化させたところ、約一週間後には、約 25-40% の HB9 陽性、Isl-1 陽性運動ニューロンを誘導することに成功した（図 2 、Hb9 陽性細胞：25.5±8.8 %、Isl-1 陽性細胞：31.1±10.9 %）。このようにして誘導した運動ニューロンは、成熟運動ニューロンのマーカーである ChAT を発現し、骨格筋との共培養により、αBTX で標識される神経筋接合部（Neuromuscular junction: NMJ）が形成されたことから、機能的な運動ニューロンが誘導されたと考えられた。

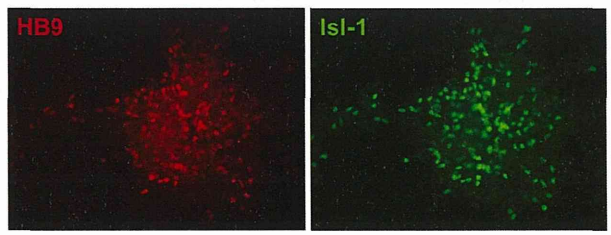

図 2　ヒト多能性幹細胞由来運動ニューロン

また、この方法を応用し、オリゴデンドロサイト前駆細胞（OPC）の増殖因子の存在下で、ニューロスフェアとして約3-6週間浮遊培養したところ、Olig2陽性、PDGFRα陽性OPCが誘導された。このニューロスフェアを約2-6週間接着分化させたところ、MBP陽性、PLP1陽性成熟オリゴデンドロサイトが誘導され、電子顕微鏡解析により、*in vitro*における髄鞘形成が確認された（Numasawa et al., *Stem Cell Reports* 2014）。

2.ヒトiPS細胞由来運動ニューロンの可視化

iPS細胞由来ニューロンを用いた解析では、固定後の免疫染色で細胞の特性を解析する手法が一般的だが、運動ニューロンを生きたまま標識・可視化することで、イメージングをはじめとした、生細胞を用いた細胞生物学的解析を行うことができる。さらにフローサイトメトリー（FACS）を用いて運動ニューロンを濃縮・純化できれば、運動ニューロン特異的な病態解析（トランスクリプトーム解析など）が可能となる。これまでに、マウスHb9の上流9kbのゲノム領域がHB9のプロモーター活性を持つことが知られており、またその上流側3.6kb、中でも*Hox*および*Pbx*結合領域を含む438bpがHb9特異的エンハンサー活性を持つことが報告されていることから（Nakano et al., *Dev Biol* 2005）、この3.6kb、438bpおよび、HoxおよびPbx結合領域含む125bpを4個タンデムにつないだエンハンサーフラグメントを作成し、β-globinのminimal promoter、および蛍光タンパクであるVenusとともにレンチウイルスベクターに組み込んで、新たなレポーターベクターを構築した（pSIN2-HB9$^{3.6kb, 438, 4x125}$-Venus）。このベクターを用いて作成したレンチウイルスを、ヒト多能性幹細胞から分化誘導した運動ニューロンに導入したところ、どのエンハンサーを用いた場合でも多くのVenus陽性細胞が観察され、その多くで運動ニューロンのマーカーであるHb9を発現していた。特にHB9^{e438}のレポーターレンチウイルスで標識した場合、Venus陽性細胞の約80%において運動ニューロンのマーカーであるHb9の発現がみられた。今後、iPS細胞由来運動ニューロンを生きたまま解析するライブセルイメージングや、FACSを用いた運動ニューロンの濃縮、純化に応用可能であると考えられる。

D.考察

ヒト多能性幹細胞から神経幹細胞や運動ニューロンを誘導する方法は複数の培養法が報告されてきたが、どの培養法も少なくとも一か月程度の培養期間を要するものが多く、疾患特異的iPS細胞を用いた解析の障壁となってきた。一方で、運動ニューロン特異的遺伝子群を導入することで、短期間で運動ニューロンを誘導する方法も報告されているが、遺伝子導入による形質変化の可能性を考慮しなくてはならない。今回、従来行われてきたDual Smad inhibitionに加え、GSK3βの阻害剤をあわせて用いることで、遺伝子導入することなく、従来よりも短時間で30-40%の運動ニューロンを誘導できる培養法を構築することができたことから、今後の運動ニューロン疾患の病態解析や薬剤スクリーニングの効率化に有用であると考えられる。

さらに、Hb9レポーターレンチウイルスを用いることで運動ニューロンを生きたままで可視化できることから、ライブセルイメージングなどによる運動ニューロン特異的な病態解析が可能になった。また、FACSなどを用いて運動ニューロンを濃縮・純化する手法を確立できれば、トランスクリプトーム解析などにおいて、運動ニューロン特異的な病態変化を捉えるために有用であると考えられる。

E.結論

ヒト多能性幹細胞から迅速・高効率に運動ニューロンを誘導する培養法を確立した。また、HB9エンハンサーを用いたレポーターレンチウイルスを作成し、運動ニューロンを生きたままで可視化する方法を構築した。今後、これらの手法を組

み合わせて、ALSなどの患者から作成したiPS細胞を用いて、運動ニューロン変性メカニズムの解析を行う予定である。

（健康危険情報）

特になし

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G.知的財産権の出願・登録状況

1. 特許取得

(1) 発明の名称：ヒト分化細胞由来多能性幹細胞由来の胚様体及び神経幹細胞培養方法
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PCT出願：PCT/JP2010/000640
申請日：2010.2.3

(2) 発明の名称：人口多能性幹細胞のクローン選択方法
発明者：岡野栄之、岡田洋平、山中伸弥、三浦恭子
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出願日：2010.5.28

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発明者：岡田洋平、岡野栄之、角田　達彦、宮　冬樹
PCT出願：PCT/JP2012/054800
出願日：2012.2.27

2. 実用新案登録

なし

3.その他

なし

Ⅲ．学会等発表実績

研究成果の刊行に関する一覧表

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雑誌

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Ⅳ. 研究成果の刊行物・別刷

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Pioglitazone suppresses neuronal and muscular degeneration caused by polyglutamine-expanded androgen receptors

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Spinal and bulbar muscular atrophy (SBMA) is a neuromuscular disease caused by the expansion of a CAG repeat in the *androgen receptor* (*AR*) gene. Mutant AR has been postulated to alter the expression of genes important for mitochondrial function and induce mitochondrial dysfunction. Here, we show that the expression levels of peroxisome proliferator-activated receptor-γ (PPARγ), a key regulator of mitochondrial biogenesis, were decreased in mouse and cellular models of SBMA. Treatment with pioglitazone (PG), an activator of PPARγ, improved the viability of the cellular model of SBMA. The oral administration of PG also improved the behavioral and histopathological phenotypes of the transgenic mice. Furthermore, immunohistochemical and biochemical analyses demonstrated that the administration of PG suppressed oxidative stress, nuclear factor-κB (NFκB) signal activation and inflammation both in the spinal cords and skeletal muscles of the SBMA mice. These findings suggest that PG is a promising candidate for the treatment of SBMA.

INTRODUCTION

Expansion of the trinucleotide CAG repeat in a coding region causes a group of neurodegenerative disorders including Huntington's disease that share several molecular pathomechanisms such as transcriptional dysregulation and mitochondrial dysfunction (1). Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motor neuron disease that exclusively affects males and is caused by the expansion of a CAG repeat in the *androgen receptor* (*AR*) gene. Spinal and bulbar muscular atrophy is characterized by proximal muscle atrophy, weakness, fasciculations and bulbar involvement (2–4). No specific treatment for this disease has been identified. Previous studies showed that polyglutamine-expanded ARs accumulate in the nuclei of motor neurons in a testosterone-dependent manner and that the pathogenic AR perturbs the transcription of diverse genes that play important roles in the maintenance of neuronal function, thereby leading to neuronal dysfunction and the impairment of retrograde axonal transport in the mouse model of SBMA (5–8).

Recent studies have shown that the pathogenesis of SBMA is a result of both neurogenic and myopathic changes. Spinal and bulbar muscular atrophy patients present with extensive motor neuron loss together with signs of muscle degeneration, including the presence of central nuclei and the degeneration of fibers (3,9). The elevated levels of serum creatine kinase (CK) also support a myopathic pathogenesis in SBMA (10,11). Histopathological analyses of muscle tissues from the SBMA mice revealed both neurogenic and myopathic features (5,12–14). In the knock-in mouse model of SBMA, muscle degeneration occurs prior to the onset of spinal cord pathology (14). Furthermore, suppression of muscle pathology ameliorates motor

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neuron degeneration in mouse models of SBMA, supporting the hypothesis that skeletal muscle is a primary target of polyglutamine-expanded-AR toxicity (13,15,16).

In the cellular model of SBMA, the accumulation of polyglutamine-expanded ARs in the presence of the relevant ligand results in mitochondrial membrane depolarization and an increase in the levels of reactive oxygen species that is blocked by treatment with the antioxidants co-enzyme Q10 and idebenone (17). Cytochrome *c* oxidase subunit Vb has been shown to interact with normal and mutant AR in a hormone-dependent manner, which may provide a mechanism for mitochondrial dysfunction in SBMA (18). Pathogenic ARs have also been shown to repress the transcription of subunits of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1), a transcriptional co-activator that regulates mitochondrial biogenesis and function; this finding suggests that polyglutamine-mediated transcriptional dysregulation is associated with mitochondrial dysfunction (17). Mitochondrial dysfunction is a common pathomechanism of polyglutamine-mediated neurodegenerative disorders, and the transcriptional repression of PGC-1 caused by mutant huntingtin is also reported in Huntington's disease (19).

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor and a ligand-activated transcription factor that regulates the expression of genes linked to a variety of physiological processes such as mitochondrial function, cell proliferation, atherosclerosis and immunity (20). Eicosanoids and 15-deoxy-prostaglandin J2 (15d-PGJ2) are naturally occurring PPARγ ligands, and thiazolidinediones, including pioglitazone (PG) and rosiglitazone, are synthetic PPARγ ligands. Thiazolidinediones elevate the expression levels of PPARγ in neuronal and non-neuronal cells and have been used to treat type II diabetes (21–23). Furthermore, transcript dysregulation of PPARγ has been shown in MN-1 cells that stably express mutant AR (AR-65Q) (17).

In the present study, we aimed to clarify whether PPARγ is involved in the pathogenesis of SBMA. Our results demonstrated that PPARγ is down-regulated in both the spinal cord and skeletal muscle of mice with SBMA and that the oral administration of a PPARγ agonist mitigates the neurodegeneration induced by polyglutamine-expanded ARs.

RESULTS

The expression level of PPARγ decreases in cellular models of SBMA

To examine the alteration in the PPARγ signaling pathway in SBMA model cells, we performed immunoblotting using neuronal cells (NSC34 cells) and muscular cells (C2C12 cells) transfected with a truncated AR (tAR-24Q or tAR-97Q). Immunoblotting analyses revealed that the expression levels of PPARγ were lower in tAR-97Q-transfected neuronal and muscular cells (tAR-97Q cells) than those in tAR-24Q-transfected cells (tAR-24Q cells), suggesting that the pathogenic AR protein bearing an expanded polyglutamine tract down-regulates the expression level of PPARγ (Fig. 1A–F). The expression levels of PPARγ were up-regulated by the administration of PG in NSC34 and C2C12 cells (Fig. 1A–F). On the other hand, the expression levels of AR were not altered by PG (Fig. 1A–F). Quantitative real-time polymerase chain reaction (RT-PCR) analyses showed that mRNA levels of PPARγ were lower in tAR-97Q cells of NSC34 and C2C12 cells than those in tAR-24Q cells; however, these levels were increased in cells treated with PG (Fig. 1A–F). Human neuroblastoma SH-SY5Y cells transfected with tAR-97Q had a lower luciferase activity under control of the *PPARγ* promoter compared with the cells transfected with tAR-24Q, indicating that the pathogenic AR inhibits the activity of the *PPARγ* promoter (Supplementary Material, Fig. S1A). SH-SY5Y cells, which stably express full-length AR-97Q also attenuated the activity of *PPARγ* promoter compared with the cells with full-length AR-24Q, and dihydrotestosterone (DHT) treatment intensifies this effect (Supplementary Material, Fig. S1B). These findings suggest that the decrease of PPARγ is associated with the nuclear accumulation of the pathogenic AR and that the transcriptional down-regulation of *PPARγ* by AR is, at least partially, hormone dependent. The expression levels of PPARγ in the spinal cords and skeletal muscles of autopsied specimens of SBMA patients had lower immunoreactivities to PPARγ than samples from control patients (Supplementary Material, Fig. S2A, B). We next investigated the effect of the increased expression of PPARγ on the cellular viability of tAR-97Q cells (Fig. 1G–L). The transient overexpression of PPARγ improved cellular viability and mitochondrial activity and attenuated cellular damage according to results of the lactate dehydrogenase (LDH) assay in both the NSC34 and C2C12 cells transfected with tAR-97Q. These findings led us to believe that the PPARγ agonist PG is a possible candidate for the treatment of SBMA.

Next, we analyzed the effects of PG on polyglutamine-mediated cytotoxicity in NSC34 and C2C12 cells by measuring cellular viability, cell death, Annexin V-positive cells, and LDH release. The transient overexpression of tAR-97Q resulted in diminished cellular viability and increased cell death, apoptotic cells and LDH release in NSC34 and C2C12 cells (Fig. 1M–T). Treatment with PG at a dose of 0.1 μM improved cellular viability of both cell lines (Fig. 1M, Q). Cell death, apoptotic cells and LDH release were also reduced by treatment with 0.1 μM PG (Fig. 1N–P, R–T). Cellular viability and cytotoxicity assays using primary cortical neurons showed similar findings (Supplementary Material, Fig. S3A, B). We further confirmed the beneficial effects of PG on hormone-dependent pathogenesis in SBMA. Pioglitazone improved the cell viability and attenuates apoptosis of a cellular model of SBMA, which stably expresses full-length AR-97Q and was treated with DHT (Supplementary Material, Fig. S4A, B). In contrast, the knock-down of PPARγ via RNAi increased LDH release and decreased mitochondrial activity in the NSC34 and C2C12 cells (Supplementary Material, Fig. S5A–C). Moreover, the effect of PG treatment was not seen when PPARγ was knocked-down, indicating that the neuroprotection by PG is dependent on PPARγ. To confirm the beneficial effects of PG on neural function, we measured the length of the axons of treated and untreated NSC34 cells (Supplementary Material, Fig. S6A, B). The axons of tAR-97Q cells were shorter than those of tAR-24Q cells, and this phenotype was improved by treatment with 0.1 μM PG.

Pioglitazone improves the histopathological findings of the spinal cord and skeletal muscle of SBMA

To test the effects of PPARγ activation *in vivo*, PG was administered to 6-week-old SBMA (AR-97Q) transgenic mice at

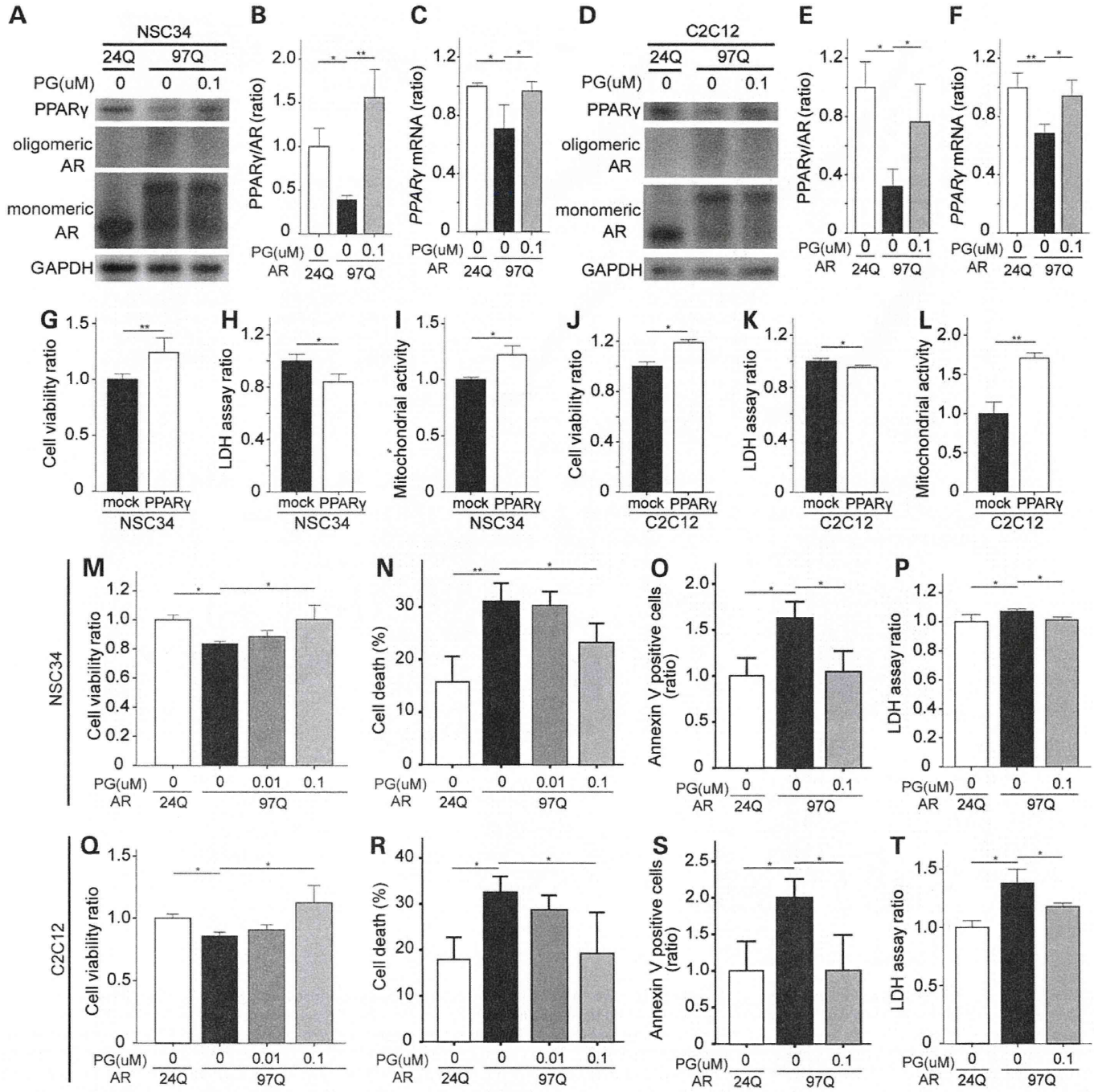

Figure 1. Pioglitazone (PG) improves the viability of cellular models of SBMA. (**A–F**) The protein and mRNA levels of *PPARγ* in NSC34 cells (A–C) and C2C12 cells (D–F) transfected with tAR-24Q or tAR-97Q and treated with or without PG. The ratio of PPARγ levels to monomeric and oligomeric AR levels (B, E). Quantitative analysis of PPARγ and AR was performed using densitometry. The *PPARγ* mRNA levels were measured using RT-PCR (C, F) ($n = 3$ per group). (**G–L**) The viability, LDH release and mitochondrial activity of NSC34 (G–I) and C2C12 cells (J–L) co-transfected with tAR-97Q and a mock or PPARγ vector ($n = 3$ per group). (**M–T**) The viability, cell death, Annexin V-positive cells and LDH release of NSC34 cells (M–P) and C2C12 cells (Q–T) transfected with tAR-24Q or tAR-97Q and treated with or without PG ($n = 6$ per group). Error bars indicate s.e.m. * $P < 0.05$ and ** $P < 0.01$ by unpaired *t*-test (G–L) or ANOVA with Dunnett's test (B, C, E, F, M–T).

concentrations of 0.01 and 0.02% in the feed until the end of analysis. No differences in feed intake were observed among the untreated, 0.01% PG-treated and 0.02% PG-treated mice at 8 weeks (data not shown). The AR-97Q mice fed the 0.02% PG-treated diet consumed 26.5 ± 1.04 mg/kg/day of PG at 8 weeks and 24.3 ± 4.44 mg/kg/day at 12 weeks. The oral administration of 0.02% PG from 6 weeks of age onward improved the body weight, performance on the rotarod task, grip power and lifespan of AR-97Q mice, although PG administered at a 0.01% dose did not improve the AR-97Q phenotype (Fig. 2A–D). Pioglitazone at 0.02% also limited the muscle atrophy and improved the stride length of the AR-97Q mice

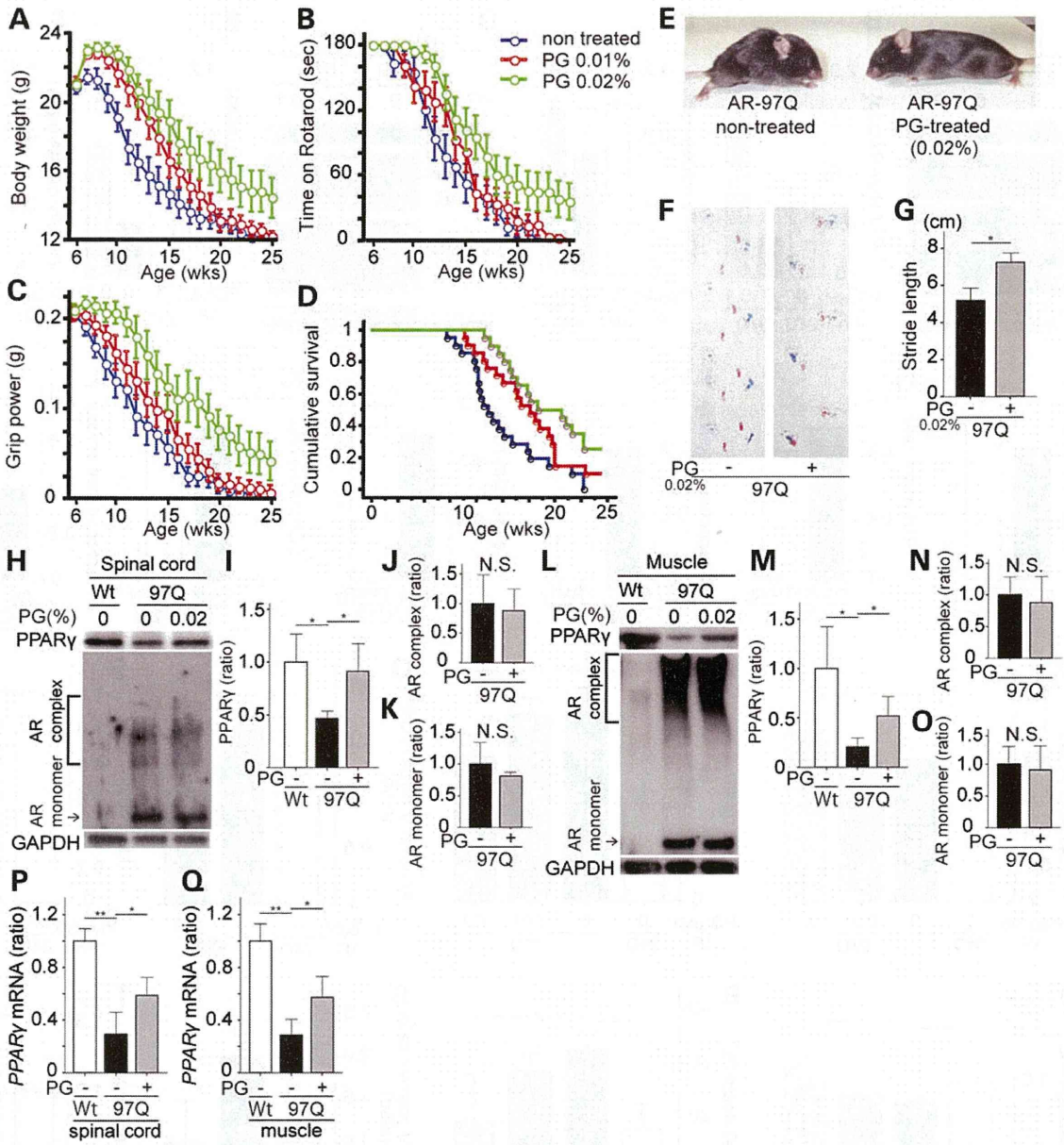

Figure 2. Pioglitazone alleviates neuromuscular phenotypes of SBMA mice. (**A**–**D**) Body weight (A), rotarod performance (B), grip power (C) and survival rate (D) of PG-treated AR-97Q mice ($n = 22$ for 0.01% PG and $n = 20$ for 0.02% PG) and untreated AR-97Q mice ($n = 21$). All parameters improved after treatment with PG at a dose of 0.02% ($P < 0.05$ at 13 weeks by ANOVA with Dunnett's test for body weight, rotarod performance and grip power; and $P < 0.005$ by log-rank test). (**E**) Muscle atrophy of 13-week-old AR-97Q mice treated with or without PG. (**F**) Footprints of 13-week-old AR-97Q mice. Front paws are shown in red, and hind paws are shown in blue. (**G**) Quantification of the footprints (13 weeks old) ($n = 3$ per group). (**H**–**Q**) Immunoblots for AR and PPARγ of the spinal cords (H–K) and skeletal muscles (L–O) of 13-week-old mice. Quantitative analysis was performed using densitometry ($n = 3$ per group). (P, Q) The mRNA levels of *PPARγ* in the spinal cord (P) and skeletal muscle (Q) of 13-week-old mice were measured using RT-PCR ($n = 3$ per group). Error bars indicate s.e.m. $^{*}P < 0.05$ and $^{**}P < 0.01$ by unpaired *t*-test (G, J, K, N, O) or ANOVA with Dunnett's test (I, M, P, Q). N.S., not significant.

(Fig. 2E–G). On the other hand, the administration of 0.02% PG had no detectable effects on the phenotypes of wild-type mice (Supplementary Material, Fig. S7A–D). There was a tendency that wild-type mice treated with PG gained weight compared with untreated wild-type mice, although the difference was not statistically significant (Supplementary Material, Fig. S7A–D). On the other hand, PG treatment increased the amount of food intake of the wild-type mice at the beginning of the treatment, but this effect faded with aging: 132.6 ± 2.5 mg/g/day of the diet without PG, and 148.5 ± 2.3 mg/g/day of the 0.02% PG-treated diet at 8 weeks ($P < 0.05$ by unpaired *t*-test); 118.2 ± 5.0 mg/g/day of the diet without PG, and 108.8 ± 10.4 mg/g/day of the 0.02% PG-treated diet at 12 weeks ($P > 0.05$).

We also examined the effects of PG when the administration was initiated after the onset of neurological symptoms. The oral administration of 0.02% PG from 8 weeks of age onward improved the body weight, performance on the rotarod task, grip power and lifespan of AR-97Q mice; however, its effects on survival were weaker than those observed when the treatment was initiated at 6 weeks of age. Specifically, the lifespan of the

mice treated at 6 and 8 weeks was 52.9 and 31.5% longer, respectively, than that of the untreated AR-97Q mice (Supplementary Material, Fig. S8A–D).

Next, we examined the biological effects of PPARγ-targeted therapy on AR-97Q mice. In agreement with the results of the cellular experiments, quantitative analyses using densitometry revealed that the expression levels of PPARγ were lower in spinal cords of untreated AR-97Q mice than those of wild-type mice; however, these levels were increased by PG treatment (Fig. 2H, I). Little difference was observed in the expression levels of AR in spinal cords of untreated and PG-treated AR-97Q mice (Fig. 2J, K). Similar findings were also observed in the skeletal muscle of AR-97Q mice (Fig. 2L–O). *PPARγ* mRNA levels were also lower in the spinal cords and skeletal muscles of untreated AR-97Q mice than in those of wild-type mice; these levels were also up-regulated in PG-treated AR-97Q mice (Fig. 2P, Q). The mRNA levels of *PGC1α* in the spinal cord were lower in untreated AR-97Q mice than those in wild-type mice. Although not significant, there was a trend that PG treatment increases the mRNA level of *PGC1α* in the spinal cord of AR-97Q mice (Supplementary Material, Fig. S9).

To investigate the pathological changes underlying the change in phenotype induced by PG, we performed immunohistochemistry on the spinal cords and skeletal muscles of wild-type, untreated AR-97Q and PG-treated AR-97Q mice. The number of 1C2-positive cells in the spinal cords and skeletal muscles was not significantly different between untreated and PG-treated AR-97Q mice (Fig. 3A–D). Immunohistochemistry for choline acetyltransferase (ChAT) in the anterior horn of the spinal cord revealed that motor neurons were atrophied in untreated AR-97Q mice but not in wild-type mice; however, the neurons were larger in PG-treated AR-97Q mice (Fig. 3E, F). Immunoreactivity to ChAT was also restored by PG treatment. Hematoxylin and eosin staining demonstrated that skeletal muscle fibers were atrophied in untreated AR-97Q mice and that PG treatment mitigated the amyotrophy in AR-97Q mice (Fig. 3G, H). Immunohistochemistry analyses using an antibody against glial fibrillary acid protein (GFAP—a marker of reactive astrogliosis) detected increased immunoreactivity in the anterior horn of the spinal cord of untreated AR-97Q mice; however, astrogliosis was attenuated by PG treatment (Fig. 3I, J). PPARγ immunohistochemistry in the spinal cord and skeletal muscle revealed that the signal intensity of PPARγ in the motor neurons and skeletal muscles was down-regulated in untreated AR-97Q mice and up-regulated by PG treatment (Supplementary Material, Fig. S10A–D). To evaluate the side effects of PG, blood was collected from the mice at 13 weeks to measure CK, aspartate aminotransferase, alanine aminotransferase and LDH serum levels. No abnormal values were observed in PG-treated AR-97Q mice, indicating that the oral administration of 0.02% PG did not induce systemic adverse effects (Supplementary Material, Fig. S11A–D). Fasting blood glucose levels were not significantly different among wild-type, untreated AR-97Q and PG-treated AR-97Q mice (Supplementary Material, Fig. S11E).

Pioglitazone suppresses oxidative stress in SBMA mice

Rosiglitazone, another PPARγ agonist, has been reported to prevent mitochondrial dysfunction and oxidative stress in mutant huntingtin-expressing cells (24). We therefore investigated the effects of PG on oxidative stress to understand the molecular basis of neuronal and muscular protection conferred by PG. We measured the mitochondrial activity of NSC34 cells and C2C12 cells that were transfected with tAR-24Q or tAR-97Q and treated with or without PG (Fig. 4A, B). The mitochondrial activity of tAR-97Q cells was lower than that of tAR-24Q cells and was improved by treatment with 0.1 μM PG.

We next measured the activity of cytochrome *c* oxidase (CCO—a marker of mitochondrial function) in the skeletal muscles of AR-97Q mice (Fig. 4C). In total, 1.5–3.0% of fibers were negative for CCO in untreated AR-97Q mice; in contrast, almost no CCO-negative fibers were found in wild-type or PG-treated AR-97Q mice ($P < 0.05$ by ANOVA with Dunnett's test for CCO-negative fibers in untreated AR-97Q mice compared with wild-type or PG-treated AR-97Q mice). To examine the expression levels of proteins related to oxidative stress, we performed immunohistochemistry using antibodies against nitrotyrosine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the spinal cords and skeletal muscles of 13-week-old mice (Fig. 4D–G). The immunoreactivity to nitrotyrosine (a marker of oxidated protein) in the motor neurons and skeletal muscles was higher in untreated AR-97Q mice than that in wild-type mice; however, the intensity was lowered after PG treatment (Fig. 4D, E). Similar effects were also observed for 8-OHdG, a marker of oxidative stress in nucleic acids (Fig. 4F, G). Specifically, PG limited the levels of nuclear 8-OHdG, particularly in motor neurons (Fig. 4F, G). We next examined the daily urinary 8-OHdG excretion of 13-week-old mice (Fig. 4H) and found that 8-OHdG excretion was higher in untreated AR-97Q mice than that in wild-type mice and was lowered by PG treatment. In a similar manner, the urinary 8-OHdG levels of SBMA patients were reported to be significantly higher than those of control patients and strongly correlated with motor function scores (25). Furthermore, immunoblot analyses using anti-4-hydroxy-2-nonenal (HNE) antibodies in the spinal cords and skeletal muscles of 13-week-old mice revealed that the expression levels of HNE, a marker of lipid peroxidation chain reactions, in the spinal cord and skeletal muscle of untreated AR-97Q mice were up-regulated and suppressed by PG treatment (Fig. 4I–K). These results suggest that PG alleviates the oxidative stress that appears to underlie the pathogenesis of SBMA.

Pioglitazone suppresses the activation of nuclear factor-κB (NFκB) pathway in SBMA mice

Nuclear factor-κB is a nuclear transcription factor that regulates the expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation and various autoimmune disorders. Previous studies have demonstrated that PG exerts anti-inflammatory effects via the attenuation of NFκB activation in the central nervous system (26,27). The augmented expression of NFκBp50 and NFκBp65 in NSC34 cells and C2C12 cells decreased cellular viability and mitochondrial activity and increased cellular damage (Supplementary Material, Fig. S12A–D). The nuclei of spinal motor neurons and skeletal muscles in autopsied specimens of SBMA patients showed increased levels of immunoreactivity to NFκB relative to control patients (Supplementary Material, Fig. S13A, B). To evaluate the activity of the NFκB pathway in cellular and mouse models of SBMA, we performed

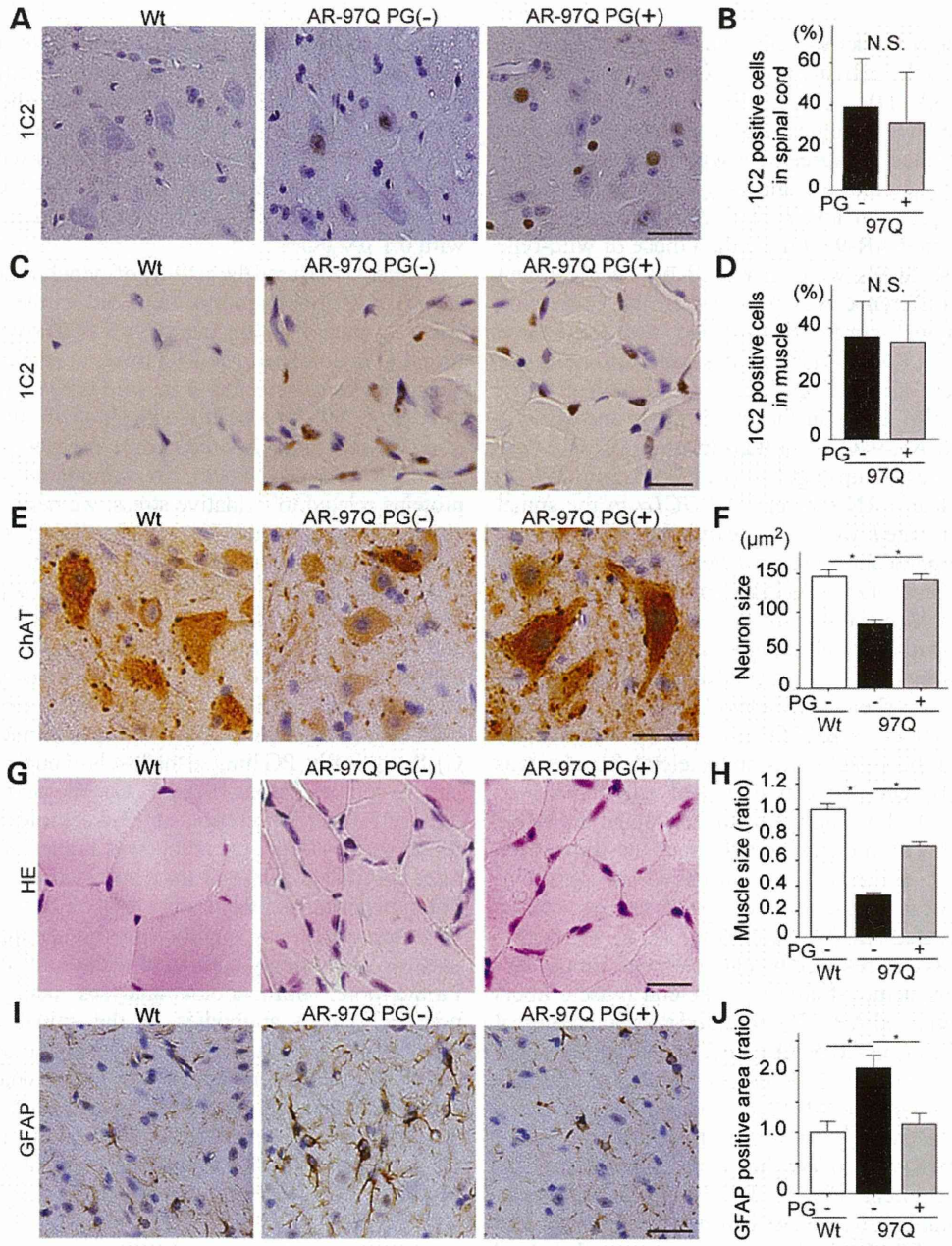

Figure 3. Effect of PG on the histopathology of SBMA mice. (**A**–**D**) Immunohistochemistry with quantitative analyses for 1C2 (an anti-polyglutamine antibody) in 13-week-old mice. (**E, F**) Anti-ChAT immunostaining of 13-week-old mice. (**G, H**) Hematoxylin and eosin staining of the skeletal muscles (G) and quantitation of muscle fiber size (H) in 13-week-old mice. (**I, J**) Immunohistochemistry with quantitative analysis for GFAP (a marker of reactive astrogliosis) in 13-week-old mice. Quantitative analyses were performed with $n = 3$ per group. Error bars indicate s.e.m. Statistical analyses were performed using the unpaired *t*-test (B, D). $^*P < 0.01$ by ANOVA with Dunnett's test (F, H, J). N.S., not significant. Scale bars: 25 μm (A, C, E, G, I).

immunoblotting for nuclear NFκBp65, cytoplasmic inhibitory protein-κ-Bα (IκBα) and phosphorylated IκBα (pIκBα) in NSC34 and C2C12 cells that were transfected with tAR-24Q or tAR-97Q and treated with or without PG. The expression levels of nuclear NFκBp65 and cytoplasmic pIκBα were up-regulated in untreated tAR-97Q cells compared with tAR-24Q cells but were down-regulated by PG treatment (Fig. 5A–H). The expression level of cytoplasmic IκBα was not significantly different among tAR-24Q, untreated tAR-97Q and PG-treated tAR-97Q cells. Anti-NFκBp65 and pIκBα immunohistochemistry also showed that the immunoreactivities of the spinal motor neurons and skeletal muscles were higher in untreated AR-97Q mice than those in wild-type mice and lower in the PG-treated AR-97Q mice (Fig. 6A–D). The alteration of NFκB signaling shown in cellular models was also observed in immunoblot analyses of spinal cords and skeletal muscles in 13-week-old wild-type untreated AR-97Q and PG-treated AR-97Q mice (Fig. 6E–L). These findings suggest that PG inhibits the activity of the NFκB pathway in both the spinal cord and skeletal muscle of AR-97Q mice.

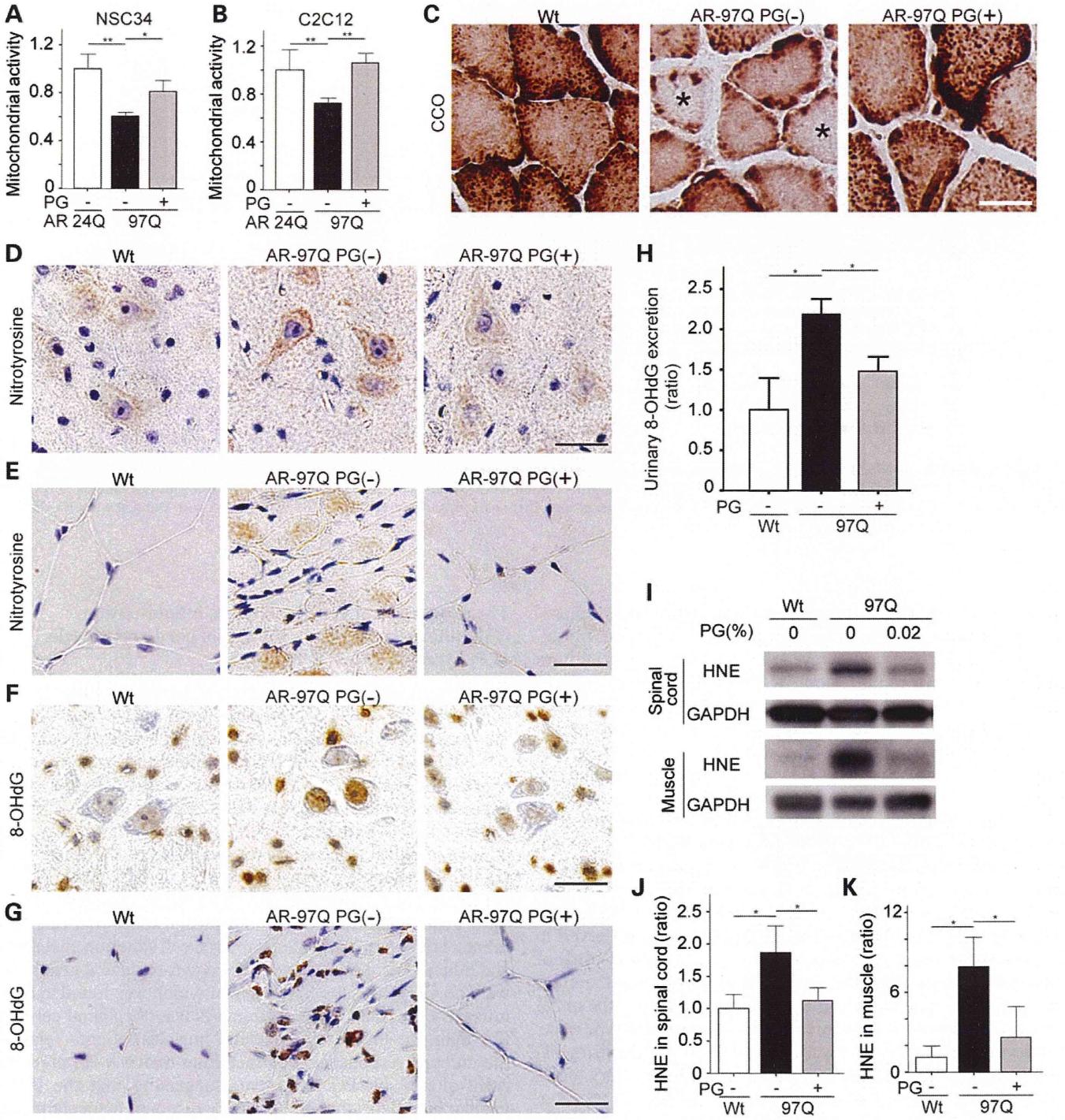

Figure 4. Effect of PG on oxidative stress in cellular and mouse models of SBMA. (**A, B**) Mitochondrial activity of the NSC34 (A) and C2C12 cells (B) transfected with tAR-24Q or tAR-97Q and treated with or without PG. (**C**) CCO (cytochrome *c* oxidase) staining of the skeletal muscles. Asterisks indicate CCO-negative muscle fibers. (**D–G**) Immunohistochemistry for nitrotyrosine and 8-OHdG in 13-week mice. (**H**) Quantitative analysis of the 24-h urinary 8-OHdG excretion of 13-week-old mice ($n = 3$ per group). Data are shown as ratio to urinary creatinine. (**I**) Immunoblots for 4-hydroxy-2-nonenal (HNE) in the spinal cords and skeletal muscles of 13-week-old mice. (**J, K**) Quantitative analysis of HNE in the spinal cords (J) and skeletal muscles (K) ($n = 3$ per group) using densitometry. Error bars indicate s.e.m. $^{*}P < 0.05$ and $^{**}P < 0.01$ by ANOVA with Dunnett's test (A, B, H, J, K). Scale bars: 25 μm (D–G).

Effects of PG on microglia

PPARs also act as master regulators governing the polarization of macrophages and microglia into 'M2' or 'alternative' activation states that suppress inflammation and promote phagocytosis and tissue repair (28,29). In contrast, microglia in 'M1' or 'classical' activation states promote the formation of the extremely toxic compound peroxynitrite, which causes damage