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Cell proliferation is a key determinant of the outcome of FOXO3a activation

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ABSTRACT

The FOXO family of forkhead transcription factors have a pivotal role in determining cell fate in response to oxidative stress. FOXO activity can either promote cell survival or induce cell death. Increased FOXO-mediated cell death has been implicated in the pathogenesis of degenerative diseases affecting musculoskeletal tissues. The aim of this study was to determine the conditions under which one member of the FOXO family, FOXO3a, promotes cell survival as opposed to cell death. Treatment of primary human tenocytes with 1 pM hydrogen peroxide for 18 h resulted in increased protein levels of FOXO3a. In peroxide-treated cells cultured in low serum media, FOXO3a inhibited cell proliferation and protected against apoptosis. However in peroxide treated cells cultured in high serum media, cell proliferation was unchanged but level of apoptosis significantly increased. Similarly, in tenocytes transduced to over-express FOXO3a, cell proliferation was inhibited and level of apoptosis unchanged in cells cultured in low serum. However there was a robust increase in cell death in FOXO3a-expressing cells cultured in high serum. Inhibition of cell proliferation in either peroxide-treated or FOXO3a-expressing cells cultured in high serum protected against apoptosis induction. Conversely, addition of a Chk2 inhibitor to peroxide-treated or FOXO3a-expressing cells overrode the inhibitory effect of FOXO3a on cell proliferation and led to increased apoptosis in cells cultured in low serum. This study demonstrates that proliferating cells may be particularly susceptible to the apoptosis-inducing actions of FOXO3a. Inhibition of cell proliferation by FOXO3a may be a critical event in allowing the pro-survival rather than the pro-apoptotic activity of FOXO3a to prevail.

1. Introduction

Cells are routinely exposed to oxidative stress as a consequence of aerobic metabolism, detoxification processes as well as through the generation of reactive oxygen species (ROS) during signalling pathway activation. Although oxidative stress is essential for maintenance of normal cell function, it can also be pathological leading to tissue degeneration.

Musculoskeletal tissues such as muscle and tendon are highly susceptible to oxidative damage-associated degenerative disease. In such diseases, increased expression of a family of transcription factors known as the FOXO forkheads has shown to have a major causal role in disease pathology. FOXOs are induced by oxidative stress and directly regulate transcription of genes encoding anti-oxidants, cell cycle regulators as well as pro-apoptotic proteins. FOXO activation can result in promotion of cell survival or cell death [1]. Which of the two occurs seems to depend on the cell environment [2–6]. Understanding why FOXO activation results in initiation of cell death rather than facilitation of cell survival under disease conditions is essential for understanding the pathogenesis of tissue degeneration.

In humans there are four FOXO family members, of which FOXO1 and FOXO3a have been most frequently implicated in oxidative stress-associated degenerative disease. In vitro studies have yielded varying results in terms of 1) which FOXO is upregulated in response to oxidative stress and 2) the outcome of FOXO activity on cell survival. The purpose of the present study is to establish the conditions under which FOXO3a is upregulated in vitro and to determine how the cell environment influences the pro-survival versus the pro-apoptotic activity of FOXO3a.

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2. Materials and methods

2.1. Cell culture

Tendon tissue was obtained from the Oxford Musculoskeletal BioBank and was collected with informed donor consent in full compliance with National and Institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki. Human tenocytes were isolated by explant culture of hamstring tendon surplus to requirements for allograft repair of ruptured anterior cruciate ligament as previously described [7]. Unless otherwise stated, tenocytes were cultured in DMEM/F12 with 5% FBS without antibiotics. Cells were passaged at 70% confluence and used up until passage 3. For experiments, cells were plated at a concentration of 50,000 cells/ml (approximately 60% confluence). Cells were allowed to adhere overnight; experimental treatments commenced on the following day.

MCF7 cells were obtained from Dr Claire Edwards, University of Oxford. MCF7s were cultured in DMEM with 10% FBS without antibiotics. Cells were passaged at 70–80% confluence and used between passages 21 and 23.

2.2. RNAi-mediated gene silencing

Cells were cultured for 18 h in serum-free DMEM-F12 with lipofectamine RNAimax (Life Technologies, Paisley UK) (1.64 μl/ml) and 36 pM siRNA (either non-targeting control catalogue number D-001810-01-05, ThermoScientific, Rockford, IL, USA or FOXO3a (catalogue no. 4392420 s5260, Life Technologies, Paisley, UK.). Lipofectamine-containing media was subsequently removed and replaced with standard growth media (DMEM-F12 containing 5% FBS) for a further 24 h before treating as appropriate for individual experiments. Success of gene knockdown was confirmed by Western Blotting 48 h post-transfection.

2.3. Adenoviral-mediated gene transduction

Cells were infected with either adFOXO3a, adFOXO3AAA (both Vector Biolabs, Philadelphia, USA; catalogue no. 1576 and 1025 respectively) or adGFP (a kind gift from Prof Jillian Cornish, University of Auckland, NZ) at MOI35 using Xtremegene HP (Roche Diagnostics Ltd, Burgess Hill, UK) following the manufacturer's instructions. Transduction efficiency was approximately 90%. Success of gene transduction was confirmed by western blotting 24 h post-infection.

2.4. Western blotting

Cells were sonicated in standard lysis buffer containing protease and phosphatase inhibitors. Proteins were quantified using the Pierce 660 protein determination assay (ThermoScientific, Rockford IL, USA) and lysates diluted as appropriate to ensure equal loading of total protein. Western blots were carried out according to standard protocols [7] and proteins visualized using Pierce WestDura detection reagents (ThermoScientific, Rockford, IL, USA) using a Chemi Doc-It imaging system with Biochemi HR camera (UVP, Upland, CA, USA). The following primary antibody was used: FOXO3a clone 75D8 (Cell Signaling Technologies, Danvers, MA, USA).

2.5. Apoptosis and cell death detection

Apoptosis was detected by measuring enzyme activity of caspase 3/7 using the Apo-One® Homogeneous Caspase 3/7 Assay following the manufacturer's instructions (Promega Corporation, Madison, USA). Cell death was confirmed using a Live/Dead Viability/Cytotoxicity staining kit following the manufacturer's instructions (Life Technologies, Paisley, UK). A Nikon Eclipse Te300 microscope equipped with a QImaging Retiga 2000R Fast 1394 camera (Nikon Corporation, Tokyo, Japan)) was used to image stained cells.

2.6. Cell proliferation

Cell proliferation was measured using the Click-it® EdU Alexa-Fluor®-555 Imaging Kit following the manufacturer's instructions (Life Technologies, Paisley, UK). Briefly, cells were incubated with the modified thymidine analogue EdU for 18 h. EdU (which is incorporated into cells undergoing DNA replication) was then fluorescently labelled with AlexaFluor-555. Cells were then stained with Hoechst 33344 in order to allow determination of total cell number. Images were captured using a Nikon Eclipse Te300 microscope equipped with a QImaging Retiga 2000R Fast 1394 camera (Nikon Corporation, Tokyo, Japan). Edu-positive cells as a percentage of total cells was calculated based on manual counts of Alexa-Fluor 555 and Hoechst 33344-labelled cells.

2.7. Statistical analysis

All experiments were repeated at least three times using different tissue donors for each experimental replicate. Results were analysed by one-way ANOVA with *post hoc* Tukey testing or by t-test if only two conditions were tested. Data were analysed using Prism 5.0b (GraphPad Software, La Jolla, California, USA). P value ≤ 0.05 was considered statistically significant. Results are expressed as mean ± SD.

3. Results

3.1. FOXO3a but not FOXO1 is induced by low level oxidative stress and protects against apoptosis induction in primary human tenocytes

Under physiological conditions, extracellular peroxide concentrations can vary from picomolar to micromolar levels depending on the source of peroxide generation [8,9]. We previously found that treatment of tenocytes with micromolar concentrations of peroxide resulted in increased protein levels of FOXO1 but not FOXO3a. Therefore, we wanted to determine if treatment with picomolar concentrations also resulted in upregulation of FOXOs. We found protein levels of FOXO3a but not FOXO1 were higher in tenocytes treated with 1 pM hydrogen peroxide for 18 h compared to untreated controls (Fig. 1A).

Level of cell proliferation was significantly lower (Fig. 1B) but level of apoptosis unchanged (Fig. 1C) in cells treated with 1 pM hydrogen peroxide for 24 h compared to untreated controls. In order to determine the effects of FOXO3a in peroxide-treated cells, we knocked down FOXO3a expression using RNAi (siFOXO3a) (Fig. 1D). We found there was no significant difference in cell proliferation in peroxide-treated siFOXO3a cells compared to control cells transfected with a non-targeting siRNA (siCntrl) indicating FOXO3a activity was responsible for the inhibition of cell proliferation following peroxide treatment (Fig. 1E). Interestingly, we found level of apoptosis was significantly higher in peroxide treated cells in which FOXO3a had been knocked down compared to peroxide-treated controls suggesting that FOXO3a had pro-survival effects (Fig. 1F).

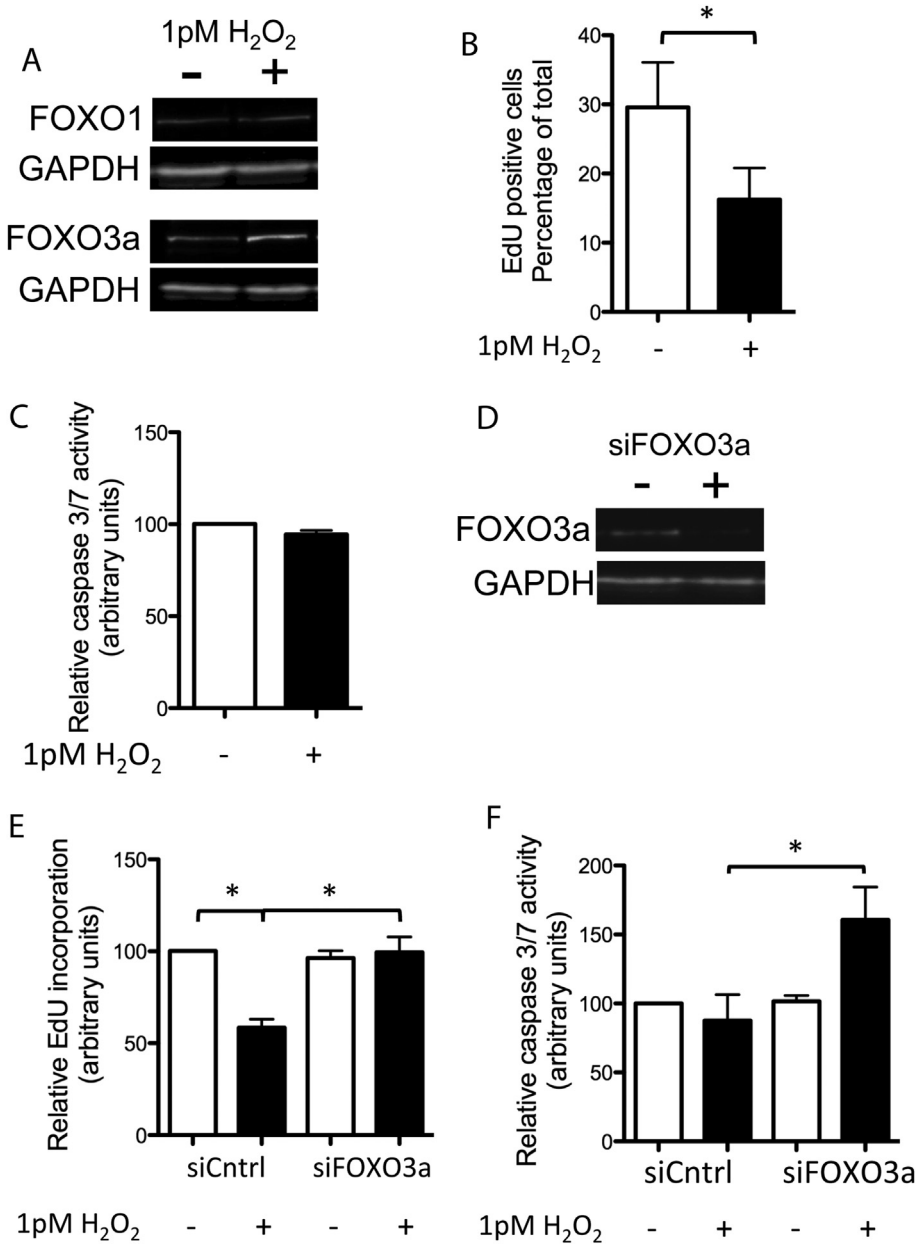

Fig. 1. FOXO3a protects against apoptosis in oxidative stress-exposed cells. **A**. Treatment of primary human tendon-derived fibroblasts (tenocytes) with 1 pM hydrogen peroxide for 8 h resulted in increased protein levels of FOXO3a but not FOXO1 as determined by western blot. **B**. The percentage of proliferating cells (cells staining positive for 5-ethynyl-2-deoxyuridine (EdU)) was significantly lower in cells treated with hydrogen peroxide for 24 h compared to untreated controls. **C**. No difference in level of apoptosis (measured using a caspase 3/7 activity assay) was apparent after 24 h of treatment with 1 pM hydrogen peroxide compared to untreated controls. **D**. Western blot showing the level of knockdown of FOXO3a protein achieved using RNAi in tenocytes. **E**. Level of cell proliferation (as determined by EdU-incorporation) was significantly higher in peroxide-treated cells transfected with siFOXO3a compared to in siCntrl cells. **F**. Caspase 3/7 activity was significantly higher in peroxide-treated cells in which FOXO3a expression had been knocked down by RNAi (siFOXO3a) compared to in peroxide-treated cells transfected with a non-targeting control siRNA sequence (siCntrl). Experiments were conducted on cells obtained from three different donors. Western blots are representative of those obtained for all 3 patient donors. Results shown are mean ± S.D. for the three experimental replicates. Differences between treatments marked with * were statistically significant ($p < 0.05$).

3.2. The pro-apoptotic effects of FOXO3a are dependent on serum levels

FOXO3a has previously been shown to induce apoptosis in a variety of different cell types including the MCF7 breast cancer cell line [10]. We wanted to determine why upregulation of FOXO3a in low dose peroxide-treated tenocytes did not result in apoptosis induction. To this end, we over-expressed FOXO3a in tenocytes using an adenoviral vector (adFOXO3a) (Fig. 2A). We found there was no difference in level of apoptosis in adFOXO3a-infected tenocytes compared to controls infected with a GFP-bearing adenoviral construct (adGFP) (Fig. 2B). To check the validity of our adFOXO3a construct, we infected MCF7 cells with our adFOXO3a vector. Consistent with the findings in previous studies [10], we found level of apoptosis was significantly higher in adFOXO3a-infected MCF7s compared to adGFP-infected controls (Fig. 2C).

MCF7 cells are typically cultured in media containing a higher percentage serum (10% FBS) than that routinely used to culture tenocytes (5% FBS). We wanted to determine if the difference in outcome of FOXO3a activation in tenocytes compared to MCF7 cells

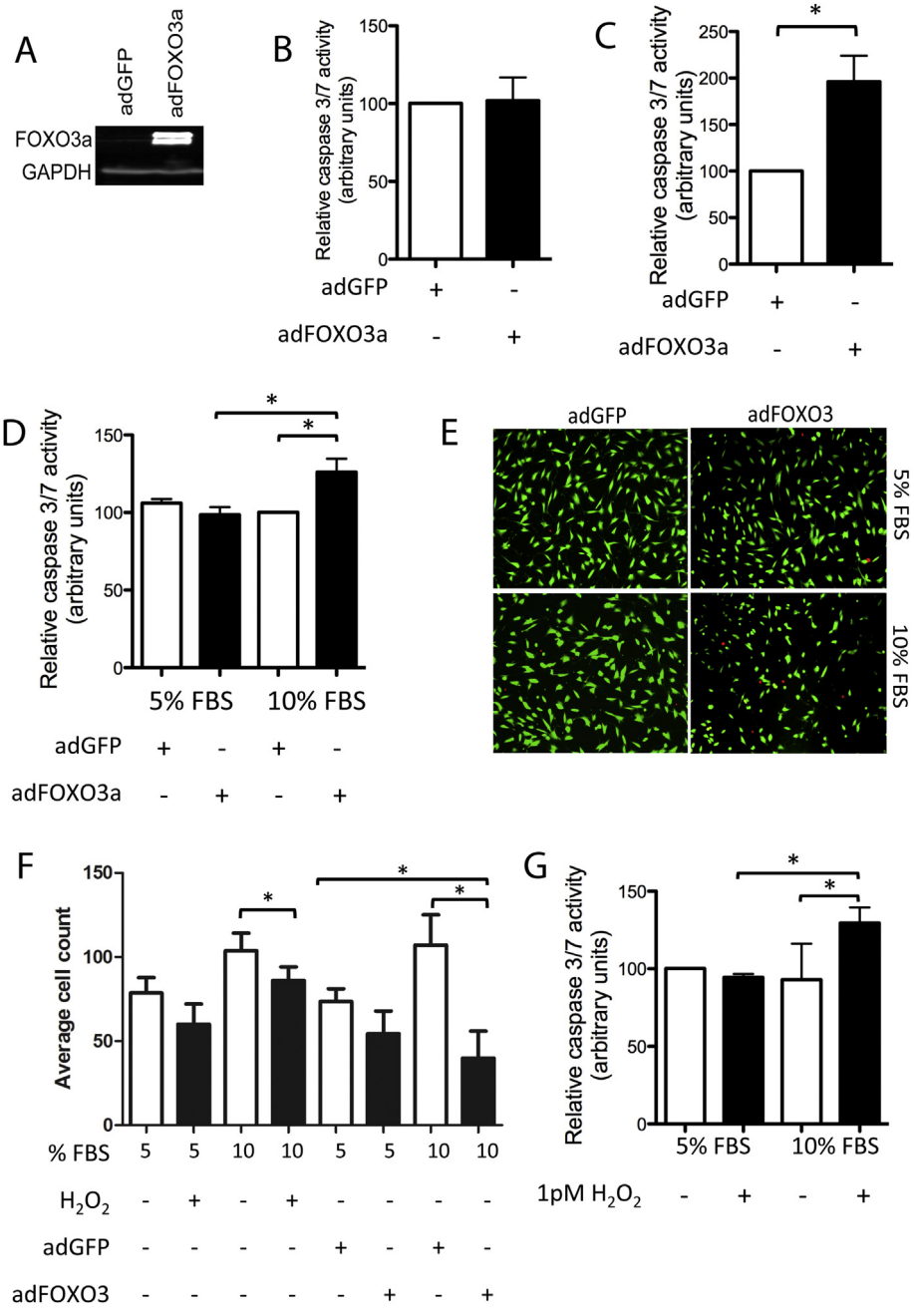

Fig. 2. The apoptosis-inducing ability of FOXO3a is dependent on serum level. **A**. Western blot showing the increase in protein level of FOXO3a obtained following infection of tenocytes with an adenoviral vector bearing a FOXO3a expression construct (adFOXO3a). Control cells were infected with a GFP-bearing adenoviral vector (adGFP). **B**. There was no difference in level of caspase 3/7 activity between cells infected with adFOXO3a and adGFP-infected controls 18 h post-infection. **C**. Caspase 3/7 activity was significantly higher in MCF7 cells infected with adFOXO3a compared to adGFP. **D**. Caspase 3/7 activity was significantly higher in primary human tenocytes cultured in media supplemented with 10% FBS compared to either adGFP-infected control cells cultured in 10% FBS or adFOXO3a-infected cells cultured in 5% FBS. Measurements were made 18 h post-infection. **E**. Photomicrographs (100×) of adGFP- or adFOXO3a-infected tenocytes cultured in 5% or 10% FBS for 24 h followed by staining with calcein AM (green, live cells) and ethidium homodimer (red, dead cells). **F**. The average number of live (calcein-AM positive) cells in cultures following 24 h of treatment with 1 pM peroxide or 24 h post infection with adFOXO3a. **G**. Caspase 3/7 activity was significantly higher in primary human tenocytes treated with 1 pM hydrogen peroxide and cultured in 10% FBS compared to either untreated controls cultured in 10% FBS or peroxide-treated cells cultured in 5% FBS. Experiments were conducted on cells obtained from three different donors. Western blot images shown are representative of those obtained for all 3 patient donors. Results shown are mean ± S.D. for the three experimental replicates. Differences between treatments marked with * were statistically significant ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reflected an inherent difference between the two cell types or was a result of the different cell culture conditions. To this end, we cultured adFOXO3a-infected tenocytes in media containing 10% serum and assessed level of apoptosis. We found caspase 3/7 activity was significantly higher in tenocytes infected with adFOXO3a cultured in 10% FBS compared to adGFP controls (Fig. 2D). By 24 h post-infection, there was evidence of ethidium homodimer incorporation (an indicator of cell death) (Fig. 2E) and a noticeable reduction in overall cell number (Fig. 2F) in adFOXO3a infected cultures.

To test if serum levels also influenced the activity of endogenous FOXO3a, we treated tenocytes cultured in 10% serum with 1 pM

peroxide. We found caspase 3/7 activity was higher in peroxide-treated cells cultured in 10% FBS compared to untreated controls (Fig. 2G) however apoptosis induction was slower than that observed in adFOXO3a-infected cells. A statistically significant increase in caspase 3/7 activity was only observed following 24 h of peroxide treatment compared to 18 h post-adFOXO3a infection. The difference in live cell number between untreated controls and peroxide-treated cells cultured in 10% FBS was much smaller than that between adGFP- and adFOXO3a-infected cells cultured under the same conditions (Fig. 2F).

3.3. The anti-proliferative effects of FOXO3a are dependent on serum levels

Next we wanted to determine if serum levels also influenced the ability of FOXO3a to inhibit cell proliferation. In adFOXO3a-infected tenocytes cultured in 5% FBS, level of proliferation was significantly lower than in adGFP-infected cells cultured under the same conditions (Fig. 3A). Although the level of proliferation was still significantly lower in adFOXO3a-infected tenocytes cultured in 10% FBS compared to adGFP-infected cells the difference was much less dramatic than in cells cultured in 5% FBS (Fig. 3A). This suggests that high serum hinders the ability of FOXO3a to inhibit cell proliferation. In support of this, we found there was no significant difference in cell proliferation between peroxide-treated cells and untreated controls cultured in 10% FBS (Fig. 3B) indicating high serum prevented the anti-proliferative effects of endogenous FOXO3a.

3.4. Inhibiting cell proliferation protects against FOXO3a-induced apoptosis

Since increasing the serum content of the media had opposing effects on the ability of FOXO3a to inhibit cell proliferation or induce apoptosis, we wanted to determine if inhibition of cell proliferation could protect against FOXO3a-induced apoptosis. We used the CDK4/6 inhibitor PD0332991 (30 nM; Sigma, Poole, UK) to inhibit cell proliferation in adFOXO3a-infected tenocytes cultured in 10% FBS (refer Supplementary information for data demonstrating the effectiveness of the inhibitor). We found level of apoptosis was significantly lower in adFOXO3a-infected cells treated with the inhibitor compared to non-inhibitor treated cells (Fig. 4A). Similarly, level of apoptosis was significantly lower in cells cultured in 10% FBS and treated with peroxide and the CDK4/6 inhibitor compared to in cells treated with peroxide alone (Fig. 4B). Next we used a Chk2 inhibitor (C3742, Sigma, Poole UK) to prevent cell cycle arrest in cells grown in 5% FBS (refer Supplementary information for data demonstrating the effectiveness of the inhibitor). We found level of apoptosis was significantly higher in adFOXO3a-infected cells cultured in 5% FBS and treated with the Chk2 inhibitor (22 nM) compared to non-inhibitor treated cells (Fig. 4C). Similarly, level of apoptosis was also significantly higher in cells cultured in 5% FBS co-treated with peroxide and the Chk2 inhibitor compared to cells treated with peroxide alone (Fig. 4D).

4. Discussion

It is widely accepted that oxidative stress has a major role in the pathogenesis of degenerative disease. However oxidative stress is a necessary consequence of aerobic life. As a result, cells utilise a variety of strategies to quench ROS and prevent or repair oxidative damage. The FOXO transcription factors upregulate expression of genes which are important components of the cellular antioxidant defence system, allowing cells to survive oxidative stress. However FOXOs can also induce apoptosis. In musculoskeletal tissues such as muscle and tendon, upregulation of FOXO-mediated apoptosis is believed to have a major causal role in the development of degenerative disease. In the present study, we sought to determine the conditions which govern whether FOXO3a activity promotes cell survival or cell death in primary human tenocytes.

We found FOXO3a protein levels were elevated in human tenocytes following treatment with low dose (1 pM) hydrogen peroxide for 18 h. Under standard growth conditions (5% FBS), FOXO3a activity in peroxide-treated cells resulted in inhibition of cell proliferation and protection against apoptosis. However in tenocytes cultured in 10% FBS, there was no reduction in cell proliferation with peroxide treatment but a significant increase in caspase 3/7 activity was observed indicating induction of apoptosis. Live cell number was also significantly lower in peroxide-treated cells cultured in 10% FBS compared to controls. A much more overt effect on live cell number was observed in tenocytes transduced to over-express FOXO3a. In these cells, exogenous FOXO3a inhibited cell proliferation when cells were cultured in 5% FBS but

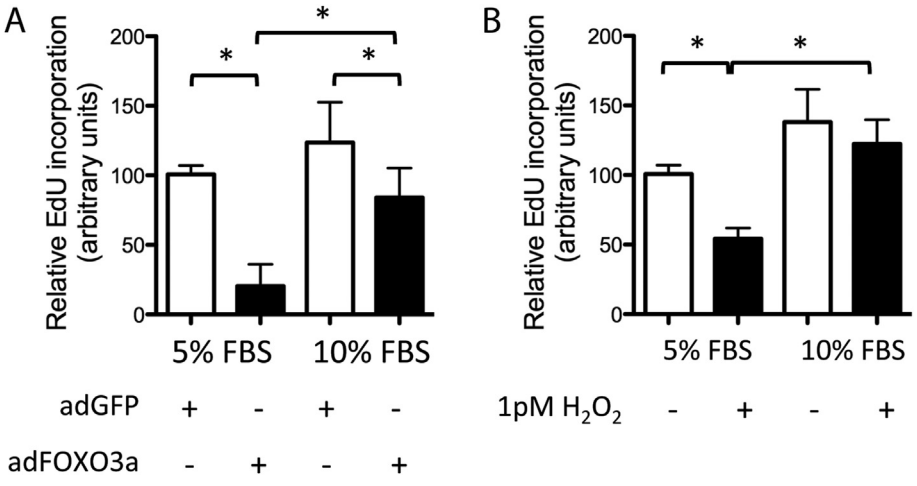

Fig. 3. The anti-proliferative activity of FOXO3a is dependent on serum level. **A**. Level of cell proliferation (as measured by EdU incorporation) was significantly lower in adFOXO3a-infected cells cultured in either 5% or 10% FBS compared to adGFP-infected controls cultured under the same conditions. However the magnitude of the difference between adFOXO3a-infected cells and adGFP-infected controls was much smaller in cells cultured in 10% FBS compared to 5% FBS. **B**. Level of cell proliferation was not significantly different between tenocytes treated with 1 pM hydrogen peroxide and untreated controls when cells were cultured in 10% FBS. Experiments were conducted on cells obtained from three different donors. Results shown are mean ± S.D. for the three experimental replicates. Differences between treatments marked with * were statistically significant ($p < 0.05$).

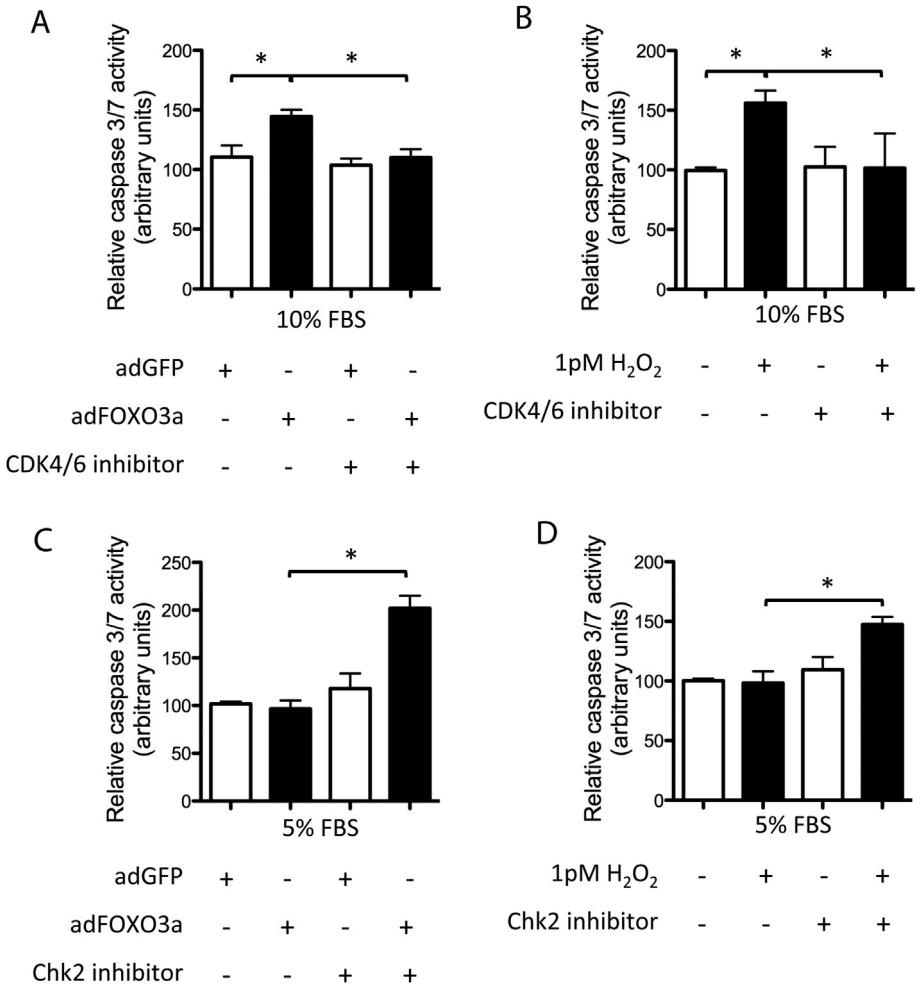

Fig. 4. Inhibition of cell proliferation protects against FOXO3a-mediated apoptosis. **A**. In cells cultured in 10% FBS, caspase 3/7 activity was significantly lower in adFOXO3a-infected cells treated with a CDK4/6 inhibitor compared to non-inhibitor treated adFOXO3a-infected cells. **B**. Similarly in cells cultured in 10% FBS, caspase 3/7 activity was significantly lower in cells co-treated with peroxide (1 pM) and a CDK4/6 inhibitor compared to cells treated with peroxide alone. **C**. In cells cultured in 5% FBS, caspase 3/7 activity was significantly higher in adFOXO3a-infecetd cells treated with a Chk2 inhibitor compared to non-inhibitor-treated adFOXO3a-infected cells. **D**. Caspase 3/7 activity was significantly higher in cells cultured in 5% FBS and co-treated with peroxide (1 pM) and the Chk2 inhibitor compared to cells cultured under the same conditions and treated with peroxide alone. Experiments were conducted on cells obtained from three different donors. Results shown are mean ± S.D. for the three experimental replicates. Differences between treatments marked with * were statistically significant ($p < 0.05$).

increased apoptosis in cells cultured in 10% FBS. Apoptosis induction in FOXO3a over-expressing cells was more rapid than in peroxide-treated cells with a significant increase in caspase 3/7 activity detectable 18 h post gene transduction compared to after 24 h of peroxide treatment. However the relative increase in caspase activity observed between adFOXO3a-infected cells and adGFP-infected controls was of a similar magnitude to that observed between peroxide treated cells and untreated controls. This may indicate that indicate that caspase activity peaked earlier than 18 h post-infection or that cell death pathways other than just apoptosis were involved in inducing cell death in adFOXO3a-infected cells.

Given the low concentration of hydrogen peroxide used in this study, the increase in apoptosis in tenocytes cultured in 10% FBS was somewhat surprising. A number of factors may have contributed to the sensitivity of tenocytes to low dose peroxide. Although cells are routinely exposed to oxidative stress in vivo, in healthy tissues the duration of this exposure is usually very transient. In this study cells were continually exposed to low dose peroxide for 18–24 h. Such prolonged exposure may have exhausted cellular defence mechanisms hence resulting in cell death. Secondly, because tenocytes are sparsely distributed throughout healthy tendon tissue, tenocyte cultures at approximately 60% confluence were used for these experiments. At this level of confluence, cells are likely to be particularly responsive to the pro-proliferative effects induced by higher FBS concentrations since very few cells would be subject to contact inhibition. This may be a crucial factor as our results indicate that proliferating cells are particularly susceptible to the pro-apoptotic activity of FOXO3a. We found that blocking the anti-proliferative activity of FOXO3a either by culturing in high serum or by using a chemical inhibitor of the Chk2 cell cycle checkpoint resulted in increased apoptosis. Interestingly this is similar to the mechanism of action of p53, another stress-induced transcription factor with both pro-survival and pro-apoptotic activity. Initiation of cell cycle arrest by p53 is crucial for enabling p53-mediated repair of stress-induced damage [11]. Like p53, FOXO3a directly regulates transcription of a number of genes involved in stress defence [12,13]. FOXO3a has also previously been shown to protect quiescent cells from oxidative stress-induced damage through the induction of genes involved in stress defence [12]. It seems likely that by inhibiting cell proliferation, FOXO3a “buys time”, preventing the replication of potentially

mutated DNA and allowing the removal of damaged cellular components whilst stress defences (such as antioxidant enzymes) work to counter the stress. If FOXO3a is unable to inhibit proliferation in stress-exposed cells, the potential for the propagation of DNA damage is high. FOXO3a has previously been shown to assist in the induction of DNA damage-induced apoptosis [14]. It follows therefore that prevention of the anti-proliferative activity of FOXO3a increases the susceptibility of cells to damage-induced apoptosis.

Increased levels of both FOXO1 and FOXO3a are common in muscle atrophy and have been implicated as having a major role in disease pathogenesis [15–18]. Increased FOXO levels have also been observed in various other degenerative conditions affecting connective tissues [19,20]. Understanding the factors which influence the ability of FOXO3a to induce cell cycle arrest may have important consequences for understanding why FOXO3a activation contributes to, rather than protects against, the development of these diseases.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.112.

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