A novel in vitro model of sarcopenia using BubR1 hypomorphic C2C12 myoblasts

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Abstract Sarcopenia is the age-related loss of skeletal muscle mass and function with adverse outcomes that include physical disability, poor quality of life, and death. The detailed molecular mechanisms remain unknown. An in vitro muscle atrophy model is needed to enable mechanistic studies. To create such a model, we employed BubR1 insufficiency which causes premature ageing in mice. Using C2C12 cells, a recognized in vitro model of the skeletal muscle cell, we obtained the BubR1 hypomorphic C2C12 (C2C12BKD) cells by using shRNA. The resulting C2C12BKD cells displayed several characteristics of the sarcopenic muscle cell. In C2C12BKD cells, formation of myotubes, assessed by analysis of fusion index, was markedly reduced as was the expression of *myogenin* and *MyoD*, two marker genes for myogenesis. Moreover, the cells showed increased expression of the muscle-specific ubiquitin ligases *Atrogin-1*

and *MuRF-1*, indicating increased protein degradation through the ubiquitin–proteasome dependent proteolytic pathway. These results suggest that C2C12BKD cells are potentially useful as a novel in vitro model of sarcopenia.

Keywords Sarcopenia · BubR1 hypomorphic C2C12 · Myogenic differentiation · Muscle-specific ubiquitin ligases

Introduction

Sarcopenia is broadly defined as the loss of skeletal muscle mass and function that occurs with ageing (Fielding et al. 2011). Decreased muscle mass and function contribute to the high incidence of accidental falls and resultant injuries observed among the elderly and can compromise quality of life (Kamel 2003). Various factors related to the development of sarcopenia include low levels of physical activity, alterations in hormone balance, loss of neuromuscular function, change in protein metabolism, poor nutrition, disease and trauma (Thomas 2007). However, the detailed molecular mechanisms causing sarcopenia remain unknown.

C2C12 cells are widely used in in vitro model systems to evaluate skeletal muscle atrophy (Shen et al. 2013; Wang et al. 2014). These cells are induced to differentiate by low-serum medium, through a process principally regulated by transcription factors

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known as the myogenic differentiation gene (MyoD) family (Langley et al. 2002). The MyoD family, including MyoD and myogenin, are also known as myogenic regulatory factors (MRFs) (Langley et al. 2002; Perry and Rudnick 2000). The activation of MRFs can lead to the fusion of myoblasts to form myotubes. Some studies have shown that MyoD family proteins are degraded by the muscle-specific ubiquitin–proteasome pathway (Bonaldo and Sandri 2013; Gumucio and Mendias 2013; Tintignac et al. 2005). Muscle Atrophy F-Box (MAFbx)/atrogin-1 and Muscle RING Finger 1 (MuRF-1) are two muscle-specific ubiquitin ligases. Upregulated atrogin-1 and MuRF-1 are responsible for the increased protein degradation occurring during muscle atrophy (Gumucio and Mendias 2013).

Dexamethasone-treated C2C12 cells, widely used to model amyotrophy, *in vitro*, show upregulated atrogin-1 and MuRF-1 (Sandri et al. 2004; te Pas et al. 2000). The synthetic glucocorticoid dexamethasone binds to the glucocorticoid receptor, activating the ubiquitin–proteasome dependent proteolytic pathway (Konagaya et al. 1986). Sarcopenia is classified as either primary or secondary. Primary sarcopenia is associated with impacts of ageing such as reduction in motor neurons, mitochondrial dysfunction and other alterations in skeletal muscle tissue, changes in the hormonal milieu and increased proinflammatory cytokines. Secondary sarcopenia is independent of age-related processes and, instead, is associated with factors such as lack of physical activity and muscle disuse, malnutrition, chronic inflammation and comorbidities (Vandewoude et al. 2012). The dexamethasone treated C2C12 cell-based system may not serve as a valid model for primary sarcopenia because it does not relate to ageing.

Another *in vitro* model, using tumour necrosis factor- α (TNF- α)-treated C2C12 cells, is associated with ceramide accumulation either by activation of sphingomyelinase or via *de novo* synthesis from palmitate. In this model, the ubiquitin–proteasome-dependent proteolytic pathway in the cells becomes activated (De Larichaudy et al. 2012; Schmitz-Peiffer et al. 1999). Moreover, ceramide-treated C2C12 cells have been used to model cellular senescence *in vitro* (Jadhav et al. 2013). These systems are not widely used to model amyotrophy, as are dexamethasone-treated cells, but nonetheless may serve as valid models for primary sarcopenia.

In vivo mouse models for amyotrophy have been produced by a variety of experimental conditions such as spinal cord isolation and transection, microgravity, hind limb suspension and denervation (Dupont-Versteegden 2006). Progeroid mice, various strains that undergo premature ageing, can also serve as *in vivo* models for amyotrophy. As an example, Baker et al. (2004) reported that BubR1 insufficiency causes premature ageing in mice and attributed this to chromosomal instability. The diaphragm muscle of BubR1-insufficient mice was sarcopenic (Greising et al. 2013). In this study, we aimed to establish an *in vitro* model of senescence-induced muscle atrophy by using BubR1 hypomorphic C2C12 cells.

Materials and methods

Culture and differentiation of C2C12 cells

Mouse myoblasts C2C12 (Yaffe and Saxel 1977b) were purchased from RIKEN BRC Cell Bank (Tsukuba, Japan). For maintenance, cells were cultured in a “growth medium” consisting of DMEM (Low glucose; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10 % FBS (Fetal Bovine Serum) (Fetal Bovine Serum; GE Healthcare, Waukesha, WI, USA) in 5 % CO₂ at 37 °C. Cell growth was monitored by cell number count and cumulative population doubling level (PDL) calculation. Population doubling (PD) gained at each passage was determined using the following formula: $PD_{[n(n-1)]} = (\text{Log } (N_n/N_{n-1}))/\text{Log } 2$, where n : passage, $n - 1$: previous passage, N_n : cell number at passage n , and N_{n-1} : cells plated at passage $n - 1$. Cumulative PDL is the sum of PDs (Böcker et al. 2008). To induce cell differentiation to myotubes, the medium was replaced with a “differentiation medium” composed of DMEM supplemented with 2 % HS (Horse Serum) (GE Healthcare) when the cells had reached 80 % confluence (Yaffe and Saxel 1977a). The differentiation medium was changed each day.

Stable cell lines expressing shRNA

After C2C12 cells were grown to 60 % confluence in 35-mm culture dishes, they were transfected with pLKO.1 (Mock) or pLKO.1 containing shRNA against *BubR1* (Sigma-Aldrich, St. Louis, MO, USA) using ESCORT IV (Sigma-Aldrich) according

to the manufacturer's instructions. Briefly, 6 µg of each plasmid were added to DMEM to a total volume of 200 µL. In parallel, 18 µL of ESCORT IV Transfection Reagent (Sigma-Aldrich) were added to 182 µL of DMEM. The diluted DNA was then added to the diluted transfection reagent and the DNA-liposome complexes were allowed to form for 45 min at room temperature. The cells were washed 2 times in PBS (phosphate-buffered saline) and 1600 µL of DMEM were then added to each dish. Four hundred µL of the DNA-ESCORT mixture were then added to each dish in a dropwise manner. Cells were incubated for 12 h, followed by additional incubation in the growth medium for 24 h. Transfected cells were treated with 6 µL/mL of puromycin (Merck KGaA, Darmstadt, Germany) for about 2 weeks, and then BubR1 hypomorphic C2C12 (C2C12BKD) cells were established. C2C12BKD cells were maintained in the growth medium including 6 µL/mL of puromycin, and the cells at high PDLs (85–206 PDLs) were used for further study. The BubR1 shRNA sequence was: 5'-CCGGGCGAATTTGTTACTCATGAACTCGAGTTTCATGAGTAACAAATTCGCTTTTT-3'.

Western blotting

Cells were washed in PBS and then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate) with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich). Samples were centrifuged at 12,000×g for 20 min at 4 °C and the total protein in the collected supernatants quantified by DCTM Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins in these samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5 % skimmed milk in T-PBS (PBS containing 0.1 % Tween20) at 4 °C overnight and then incubated with either BubR1 (1:1000) or β-actin (1:3000) antibody (Sigma-Aldrich) for 1 h at room temperature. Membranes were then washed in T-PBS and incubated with horseradish peroxidase conjugated secondary antibody (Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. After membranes were washed again with T-PBS, horseradish peroxidase activity was detected using ImmunoStar Zeta (Wako Pure Chemical Industries) and a Chemi Doc XRS-J digital densitometer (Bio-Rad Laboratories).

Quantitative real time PCR

Total RNA was extracted from cells using TRIzol[®] reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 0.5 µg of total RNA using ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan). The used RT program was: 15 min at 37 °C; 5 min at 50 °C; 5 min at 98 °C. Afterward, real time PCR was performed in a total reaction volume of 20 µL containing 1.5 µL of cDNA, 1.0 µL of each gene-specific primer (10 µM), 10.0 µL of SYBR[®] Premix Ex TaqTM (Takara, Tokyo, Japan) and 6.5 µL of distilled water, using a Thermal Cycler Dice Real Time System TP800 (Takara). The real time PCR condition for *36B4*, *p19^{Arf}*, *p21^{Cip1}*, *MuRF-1* and *Atrogin-1* included an initial step at 95 °C for 30 s; 40 cycles at 95 °C for 5 s, 57 °C for 10 s and 72 °C for 20 s. For *p16^{INK4a}*, *TNF-α* and *IL-6*, the real time PCR condition included an initial step at 95 °C for 30 s; 40 cycles at 95 °C for 5 s, 57 °C for 20 s and 72 °C for 1 min. A melting curve analysis was performed at the end of each reaction at temperatures between 50 and 90 °C. All signals were normalized to the signal for *36B4*. The primer sequences used for PCR are shown in Table 1.

Semiquantitative RT-PCR

The cDNA was synthesized as described above. PCR was performed in a total reaction volume of 20 µL containing 1.0 µL of cDNA, 0.2 µL of each gene-specific primer (50 µM), 0.1 µL of TaKaRa Ex Taq (Takara), 2.0 µL of 10 × Ex Taq buffer, 1.6 µL of dNTP mixture and 14.9 µL of distilled water, using the MJ MiniTM Personal Thermal Cycler (Bio-Rad Laboratories). The semiquantitative RT-PCR condition included an initial step at 95 °C for 30 s; 27, 30 and 35 cycles (*36B4*, *MyoD* and *myogenin*, respectively) at 98 °C for 10 s, 57 °C for 30 s and 72 °C for 1 min; and, finally, 1 cycle at 72 °C for 5 min. PCR products were subjected to 2.0 % agarose gel electrophoresis. Data were collected with a FAS-III UV-image analyser (Takara). Semiquantitative RT-PCR was performed using *36B4* as the housekeeping gene. The primer sequences used for PCR are shown in the Table 1.

Table 1 Primer sequences used for quantitative real time PCR and semiquantitative RT-PCR

Gene symbol	Sequence (5′–3′)	
<i>36B4</i>	Forward	AGATTCGGGATATGCTGTTGGC
	Reverse	TCGGGTCCTAGACCAGTGTTC
<i>p16^{INK4a}</i>	Forward	CGTACCCCGATTACAGGTGAT
	Reverse	TTGAGCAGAAGAGCTGCTACGT
<i>p19^{Arf}</i>	Forward	AAGAAGTCTGCGTCGGCGAC
	Reverse	AGTACCGGAGGCATCTTGGACA
<i>p21^{Cip1}</i>	Forward	ACAGGAGCAAAGTGTGCCGTTGT
	Reverse	GCTCAGACACCAGAGTGCAAGACA
<i>MyoD</i>	Forward	CGTGGCAGCGAGCACTAC
	Reverse	TGTAATCCATCATGCCATCAGA
<i>myogenin</i>	Forward	CAGGAGATCATTTGCTCG
	Reverse	GGGCATGGTTTCGTCTGG
<i>MuRF-1</i>	Forward	GCTGGTGGAAAACATCATTGACAT
	Reverse	CATCGGGTGGCTGCCTTT
<i>Atrogin-1</i>	Forward	CACATTCTCTCCTGGAAGGGC
	Reverse	TTGATAAAGTCTTGAGGGGAA
<i>TNF-α</i>	Forward	GATCGGTCCCCAAAGGGATG
	Reverse	TGAGGGTCTGGGCCATAGAA
<i>IL-6</i>	Forward	GCTACCAAACTGGATATAATCAGGA
	Reverse	CCAGGTAGCTATGGTACTCCAGAA

SA- β -galactosidase staining

C2C12 cells were stained for senescence associated- β -galactosidase (SA- β -galactosidase) activity according to the method of Debacq-Chainiaux et al. (2009). Briefly, cells were fixed with 4 % paraformaldehyde and washed once with PBS. Cells were incubated with a staining solution (1 mg/mL X-Gal in dimethyl formaldehyde, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 150 mM NaCl and 40 mM citric acid/phosphate pH 6.0) for 12 h at 37 °C. After staining, cells were washed with PBS and SA- β -galactosidase activity, indicated by a blue stain, was visualized with an Axiovert 40 CFL (Carl Zeiss, Oberkochen, Germany) and image-analysed with the Axio Vision (Carl Zeiss).

Fusion index

The fusion index was calculated according to the modified method of Nakajima et al. (2006). C2C12 cells undergoing differentiation were fixed with 4 % paraformaldehyde for 30 min at 37 °C and stained with DAPI solution (Wako Pure Chemical Industries). Three different images were captured from random fields with

an Axiovert 40 CFL (Carl Zeiss) and analysed with the Axio Vision (Carl Zeiss). The numbers of nuclei in myotubes and total nuclei were counted in each field. The fusion index was calculated as the percentage of total nuclei incorporated into myotubes.

Statistical analysis

All results are expressed as the mean $\pm$ standard deviation (SD). Student's *t* test was used to evaluate the differences between the means of the results. Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).

Results and discussion

BubR1 hypomorphic C2C12 cells do not reach senescence

Mutant mice expressing low levels of the mitotic checkpoint protein BubR1 undergo premature separation of sister chromosomes and develop progressive aneuploidy along with various progeroid phenotypes

Fig. 1 BubR1 expression and senescence markers in C2C12BKD cells. C2C12BKD cells were generated from C2C12 cells using the BubR1 knockdown protocol described in Materials and methods. Control C2C12 cells (“Mock”) were mock transfected. **a** BubR1 by western blotting with β -actin as a sample loading control (8.2, 107.7 and 202.7 PDLs). **b** Cell growth. Cells (3.0×10^5 cells per dish) were seeded into each 35-mm dishes (95.0 PDL). Cells were trypsinized and counted at the indicated times after seeding. Data are the mean $\pm$ SD ($n = 4$). **c–e** Relative expression of CDK inhibitors using qRT-PCR analysis. C2C12BKD ($p16^{INK4a}$; 200.8 PDL, $p19^{Arf}$ and $p21^{Cip1}$; 130.7 PDL) and Mock cells were collected after 2 days of culture in the growth medium. All signals were normalized to the signal for $36B4$. In the figure, these values are presented as relative expression values to corresponding Mock control. Data are the mean $\pm$ SD ($n = 4$). NS, Not Significant (the p value obtained in the Student’s t -test results). **f** Phase-contrast images of SA- β -galactosidase activity. Cells were seeded at equivalent densities (3.0×10^5 cells per dish) onto 35-mm dishes (123.6 PDL). After 24 h, cells were stained for SA- β -galactosidase as described in Materials and Methods. Neither cell exhibited positive staining. The size of the scale bar is 50 μ m

response to activated oncogenes (oncogene-induced senescence) or on exposure of cells to DNA-damaging insults or other stresses (Campisi and d’Adda di Fagagna 2007; Collado et al. 2007). Induction of cell senescence is typically associated with increased cell cycle inhibitory proteins (e.g. $p16^{INK4a}$, $p21^{Cip1}$, and $p53$), SA- β -galactosidase, senescence-associated DNA-damage foci (SDFs) and senescence-associated heterochromatin foci (SAHFs) (Campisi and d’Adda di Fagagna 2007). Previous studies have shown that mouse embryonic fibroblasts (MEFs) isolated from BubR1 hypomorphic mice show gradually increased expression of cell cycle inhibitory genes, particularly $p16^{INK4a}$, most evident in cultures at passage 8 or higher (Baker et al. 2004). However, even at PDLs greater than 95.0, C2C12BKD cells showed no significant changes in cell growth or expression of cell cycle inhibitory genes (Fig. 1b–e). In a recent report, high ceramide concentrations induced senescence of C2C12 cells, along with increased SA- β -galactosidase staining (Jadhav et al. 2013), while C2C12BKD cells did not stain positively (Fig. 1f, Supplementary Figure 1). SA- β -galactosidase is associated with cellular senescence, but it is not completely specific (Debacq-Chainiaux et al. 2009). Indeed, skeletal muscles of BubR1 hypomorphic mice did not stain positive for SA- β -galactosidase but expressed high levels of several senescence-associated genes and lost proliferative potential (Baker et al. 2008) (Table 2: age related phenotypes).

including sarcopenia (Baker et al. 2004). To produce an in vitro model of sarcopenia, we transfected C2C12 cells with a shRNA vector designed for knockdown of the *BubR1* gene. Western blot analysis showed that BubR1 hypomorphic C2C12 cells, which we designated C2C12BKD cells, were generated (Fig. 1a). Even if PDL of C2C12BKD cells exceeded 200, the protein expression of BubR1 was stably low. Cellular senescence, a state of irreversible withdrawal of cells from the proliferative pool, occurs on exhaustion of the proliferative lifespan (replicative senescence), in

Synthetically, our data suggest that C2C12BKD cells did not reach senescence.

BubR1 hypomorphic C2C12 cells show impaired myogenic differentiation

An important factor in sarcopenia is the imbalance of protein metabolism toward decreased protein synthesis or increased protein degradation (Bonaldo and Sandri 2013; Gumucio and Mendias 2013). C2C12 cells are induced to differentiate into multinuclear myotubes by a low serum “differentiation medium” (Yaffe and Saxel 1977b). To examine the effect of BubR1 underexpression on the muscle differentiation of C2C12 cells, the growth medium (10 % FBS) was switched to the differentiation medium (2 % HS). C2C12 cells, as expected, were substantially induced to form multinuclear myotubes by day 7 in the differentiation medium. However, C2C12BKD cells showed no detectable myotube formation even after 11 days (Fig. 2a). Figure 2b illustrates the significant difference in fusion index between C2C12 and C2C12BKD cells from day 7 onward. In the two other in vitro models, using TNF- α - and dexamethasone-treated C2C12 cells, myotube formation was decreased in the differentiation medium but was not absent (Langen et al. 2001; Sharples et al. 2010; te Pas et al. 2000) (Table 2: myotube formation). In contrast, in C2C12BKD cells myotube formation was essentially abolished. Therefore, C2C12BKD cells provide a novel, potentially valuable model for sarcopenia.

A decline in essential MRFs, including MyoD and myogenin, is known to contribute to skeletal muscle loss (Ma et al. 2012). Expression of both *MyoD* and *myogenin* was decreased in C2C12BKD cells after 11 days in the differentiation medium (Fig. 2c). These data are consistent with observations in other in vitro models (Langen et al. 2001; Sharples et al. 2010; Strle et al. 2004; te Pas et al. 2000) and indicate that the reduced expression of these markers is responsible for the loss of differentiation capacity in C2C12BKD cells (Table 2: differentiation markers). Baker et al. reported that expression of *p16^{Ink4a}* in BubR1 hypomorphic mice plays a key role in the development of age-related disorders including sarcopenia (Baker et al. 2008, 2011). Expression level of *p16^{Ink4a}* in skeletal muscle derived from BubR1 hypomorphic mice was approximately 17 times higher than for the wild type (Baker et al. 2011). Therefore, we expected that *p16^{Ink4a}*

expression was increased in C2C12BKD cells. In fact, *p16^{Ink4a}* expression tended to increase, but there was no statistical difference (Fig. 1c). Collectively, our data suggest that C2C12BKD cells lost differentiation potency in the style which was not associated with *p16^{Ink4a}*. This point is one of the marked differences with BubR1 hypomorphic mice model.

The ubiquitin–proteasome pathway is activated in C2C12BKD cells

Muscle atrophy is a tightly controlled process involving many signalling pathways. In particular, the ubiquitin–proteasome pathway plays an important role in muscle protein degradation (Gumucio and Mendias 2013). MuRF-1 and Atrogin-1 are two muscle-specific ubiquitin ligases that are important regulators of ubiquitin-mediated protein degradation in skeletal muscle (Gumucio and Mendias 2013). To investigate whether the ubiquitin–proteasome system was activated in C2C12BKD cells, expression levels of *MuRF-1* and *Atrogin-1* were determined using qRT-PCR. Expression of both genes was significantly increased in C2C12BKD cells after 11 days in the differentiation medium (Fig. 3a, b). These results indicate increased proteolysis through an ubiquitin–proteasome dependent proteolysis pathway in C2C12BKD cells. In dexamethasone- and TNF- α -treated C2C12 cells, there is also increased expression of muscle-specific ubiquitin ligase (De Larichaudy et al. 2012; Hemdan et al. 2009) (Table 2: muscle-specific ubiquitin ligase). The expression of these genes in BubR1 hypomorphic mice has not been reported. Based on our observations, however, we speculate that BubR1 hypomorphic mice also have increased muscle protein proteolysis through an ubiquitin–proteasome dependent proteolysis pathway.

Inflammation may negatively influence skeletal muscle. In an observational study of more than 2000 persons, TNF- α , a proinflammatory cytokine, showed an association with declines in muscle mass and strength (Schaap et al. 2009). TNF- α is believed to contribute to muscle wasting by activating protein degradation via the ubiquitin–proteasome pathway (Caron et al. 2011). Such chronic inflammation-induced protein degradation might mediate the loss of differentiation potential in C2C12BKD cells. Indeed, addition of TNF- α prevents C2C12 myoblasts differentiation through activation of nuclear factor-

Table 2 Comparative characteristics of in vivo and in vitro sarcopenia models

	In vivo model			In vitro model		
	Normal senescent mice	Tumour cell-transplanted mice (ceramide accumulation)	Dexamethasone-treated mice	BubR1 hypomorphic mice	Ceramide-treated C2C12 cells	TNF- α -treated C2C12 cells
<i>Age related phenotypes</i>						
Cyclin-dependent kinase inhibitors	Increased expression ^a (<i>p16</i> , <i>p19</i> , <i>p21</i> , <i>p27</i>)	NR	NR	Increased expression ^{d, e} (<i>p16</i> , <i>p19</i> , <i>p21</i> , <i>p53</i>)	Increased expression ^g (<i>p21</i> , <i>p53</i>)	NR
SA- β -galactosidase	Positive ^a	NR	NR	Negative ^e	Positive ^g	NR
<i>Muscle atrophy phenotypes</i>						
Differentiation potential	Decreased ^a	Decreased ^b	Decreased ^c	Decreased ^f	NR	NR
Cross-sectional areas	NR	NR	NR	NR	NR	NR
Myotube formation	NR	NR	NR	NR	NR	Decreased myotubes ^k
Differentiation markers (<i>MyoD</i> , <i>myogenin</i>)	NR	NR	No significant difference ^c (<i>MyoD</i>)	NR	Decreased expression ^b (<i>MyoD</i> , <i>myogenin</i>)	Decreased expression ^k (<i>MyoD</i> , <i>myogenin</i>)
Muscle-specific ubiquitin ligases (<i>Atrogin-1</i> , <i>MuRF-1</i>)	NR	Increased expression ^b (<i>Atrogin-1</i> , <i>MuRF-1</i>)	Increased expression ^c (<i>MuRF-1</i>)	NR	NR	Increased expression ^l (<i>Atrogin-1</i> , <i>MuRF-1</i>)
Proinflammatory cytokines (TNF- α , IL-6)	Increased expression ^a (TNF- α , IL-6)	NR	NR	Increased expression ^f (IL-6)	NR	NR
						Increased expression (TNF- α , IL-6)

NR not reported

^a Sousa-Victor et al. (2014); ^b De Larichaudy et al. (2012); ^c Noh et al. (2014); ^d Baker et al. (2004); ^e Baker et al. (2008); ^f Greising et al. (2013); ^g Jadhav et al. (2013); ^h Strle et al. (2004); ⁱ Langen et al. (2001); ^j Sharples et al. (2010); ^k te Pas et al. (2000); ^l Hemdan et al. (2009)

Fig. 2 Differentiation capacity of C2C12BKD cells. Mock and C2C12BKD cells were generated and induced to differentiate as described in Materials and Methods. **a** Representative micrographs of myogenesis in Mock and C2C12BKD cell (184.2 PDL) cultures at various times after transfer to the differentiation medium. The size of the scale bar is 50 μm . **b** Fusion index, representing the proportion of total nuclei found in

myotubes, was calculated as described in Materials and methods. Data are the mean $\pm$ SD ($n = 3$). **c** Relative expression of differentiation markers using semiquantitative RT-PCR analysis. C2C12BKD (204.4 PDL) and Mock cells were collected after 11 days of culture in the differentiation medium. One representative gel is shown

kappa B (Langen et al. 2001; Sharples et al. 2010). However, increased expression of proinflammatory cytokines has not been reported on C2C12 models for muscle loss (Table 2: proinflammatory cytokines). Expression of the proinflammatory cytokines *TNF- α* and *interleukin 6 (IL-6)* was increased significantly in C2C12BKD cells (Fig. 4a, b).

Senescent cells are known to secrete proinflammatory factors, exhibiting the senescence-associated secretory phenotype (SASP) (Salama et al. 2014). Interestingly, increased expression of proinflammatory cytokines was observed in both in vivo (Baker et al. 2013) and the in vitro BubR1 hypomorphic models as well as in normal senescent mice (Sousa-

Victor et al. 2014) (Table 2: proinflammatory cytokines). While C2C12BKD cells showed no senescence markers (Fig. 1c–f), the expression patterns of proinflammatory factors seen in C2C12BKD cells (Fig. 4a, b) raise the possibility that these cells can exert autocrine and/or paracrine effects similar to those associated with SASP. These proinflammatory factors might mediate muscle atrophy in these models via the ubiquitin–proteasome pathway. C2C12BKD cells might, therefore, be a uniquely valuable in vitro system for studying the relationship between chronic inflammation and sarcopenia.

An in vitro model will be essential for the discovery of novel agents for prevention of sarcopenia. Because

Fig. 3 Expression of muscle-specific ubiquitin ligases related genes in C2C12BKD cells using qRT-PCR analysis. C2C12BKD (204.4 PDL) and Mock cells were collected after 11 days of culture in the differentiation medium. Total RNA from the cells was used for qRT-PCR analysis, and mRNA

expression levels of *MuRF-1* (a) and *Atrogin-1* (b) were evaluated. All signals were normalized to the signal for *36B4*. These values are presented as normalized to that of the corresponding Mock control. Data are the mean $\pm$ SD ($n = 4$)

Fig. 4 Expression of proinflammatory cytokines related genes in C2C12BKD cells using qRT-PCR analysis. C2C12BKD (130.7 PDL) and Mock cells were collected after 2 days of culture in the growth medium. Total RNA from the cells was used for qRT-PCR analysis, and mRNA expression levels of

TNF-α (a) and *IL-6* (b) were evaluated. All signals were normalized to the signal for *36B4*. These values are presented as relative expression values to the corresponding Mock control. Data are the mean $\pm$ SD ($n = 4$)

many factors contribute to the development of sarcopenia, a variety of experimental model systems will be needed to thoroughly assess potential therapeutic interventions. We anticipate that C2C12BKD cells will play an important role in this effort as a valuable in vitro sarcopenia model.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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