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Membrane-bound delta-like 1 homolog (Dlk1) promotes while soluble Dlk1 inhibits myogenesis in C2C12 cells

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ABSTRACT

Delta-like 1 homolog (*Dlk1*) is important in myogenesis. However, the roles of different Dlk1 isoforms have not been investigated. In C2C12 cell lines producing different Dlk1 isoforms, membrane-bound Dlk1 promoted the hypertrophic phenotype and a higher fusion rate, whereas soluble Dlk1 inhibited myotube formation. Inversed expression patterns of genes related to myogenic differentiation further support these phenotypic changes. In addition, temporal expression and balance between the Dlk1 isoforms have a regulatory role in myogenesis *in vivo*. Collectively, Dlk1 isoforms have distinctive effects on myogenesis, and its regulation during myogenesis is critical for normal muscle development.

1. Introduction

Myoblasts undergo proliferation followed by differentiation into mononucleated myocytes to form the myotube by fusing to each other or to an existing myotube. The balance between differentiation and reserving the myoblast is important for maintaining muscle mass. Four myogenic regulatory factors (MRFs), myogenic differentiation (*MyoD*), myogenic factor 5 (*Myf5*), myogenin (*Myog*), and *Mrf4*, and the inhibitory factors paired box 7 (*Pax7*), *Notch*, and bone morphogenetic protein 4 (*Bmp4*), are known to regulate this balance [1–9].

Delta-like 1 homolog (*DLK1*) is considered to be a candidate gene causing muscle hypertrophy in callipyge sheep, and overexpression of sheep *DLK1*-C2 in a transgenic mouse model had the same effect of increasing muscle mass [10–12]. Full-length DLK1 is a glycoprotein consisting of six EGF-like domains, a protease-sensitive juxtamembrane domain, and a transmembrane domain. In the mouse, there are several splicing isoforms of *Dlk1* mRNA, and the resulting protein isoforms can be distinguished by whether the resulting juxtamembrane domain is retained or not [13,14]. The A and B isoforms have the juxtamembrane domain and can produce the soluble form of Dlk1 after being cleaved by a tumor necrosis factor-alpha converting enzyme (TACE) [15]. The other forms without juxtamembrane domain, C, C2, D, and D2, are not cleaved and remain on the cell membrane [14]. The number of alternative splicing isoforms of *Dlk1* varies among species, and expression levels and patterns of the alternative splicing isoforms are altered depending on the development stage and age [10,11,16–19]. *Dlk1*-null mice have a phenotype of growth retardation, obesity, and skeletal malformation, whereas overexpression of soluble Dlk1 causes inhibition of preadipocyte differentiation and metabolic abnormality [20,21]. Notably, recent studies showed the critical role of *Dlk1* in myogenesis and muscle regeneration [22–24].

Although *Dlk1* has been found in several isoforms and cleavage products *in vivo*, the function of these isoforms in regulating myogenesis has not been studied. In this study, the function of representative mouse Dlk1 isoforms in muscle development were investigated in C2C12 cells, using 6 EFG as a soluble Dlk1, C2 form as a membrane-bound Dlk1, and A form as an intermediate form.

Abbreviations: Dlk1, delta-like 1 homolog; MRF, myogenic regulatory factor; MyoD, myogenic differentiation; Myf5, myogenic factor 5; Myog, myogenin; Pax7, paired box 7; Bmp4, bone morphogenetic protein 4; Mstn, myostatin; CLPG, callipyge mutation; EGF, epidermal growth factor; TACE, tumor necrosis factor-alpha converting enzyme; HA, hemagglutinin; RT, reverse transcription; RT-qPCR, reverse-transcription quantitative PCR; Rplp0, ribosomal protein, large, P0; Fst, follistatin; Cav3, caveolin3; Nog, Noggin; Murf1, muscle-specific RING finger protein 1; Foxo3, forkhead box O3

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2. Materials and methods

2.1. Vector construction

The coding sequences of mouse *Dlk1*-A and -C2 isoforms were amplified from mouse cDNA by PCR with the primer set mDlk1-F and mDlk1-R (Suppl. Table 1) and cloned into the pQCXIP retroviral vector (Clontech, Mountain View, CA). Vectors containing hemagglutinin (HA)-tagged *Dlk1* were constructed by adding HA-tag sequences at the 3′ end using PCR with a mDlk1-R-HA primer [25]. The 6 epidermal growth factor (EGF) with a C-terminal HA-tag was amplified by PCR with the primer set mDlk1-F and 6EGF-R-HA and was cloned into the pQCXIP vector. The pQCXIP retroviral vectors containing no gene (empty vector, EV) or each isoform of *Dlk1* were used to produce the retrovirus for infecting the C2C12 cells.

2.2. C2C12 cell line establishment and culture

The C2C12 mouse myoblast line (ATCC, Manassas, VA) was maintained with DMEM (Gibco, Grand Island, NY) containing 10% FBS (Gibco) and 1× penicillin–streptomycin (Gibco). To establish cell lines for control or expressing the Dlk1 isoforms, C2C12 cells were infected by retroviruses encoding no gene (EV) or each *Dlk1* isoform, and subjected to selection with 2.5 μg/ml of puromycin (Clontech) for 10 days by subculturing before confluency. For differentiation, when the cells reached 90% confluence the medium was replaced with a differentiation medium containing 2% horse serum (Gibco) in DMEM. Half of the medium was replaced with fresh differentiation medium every 2 days.

2.3. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. Muscle tissues were collected from fetuses of the ICR (CD-1®) outbred line (Harlan Laboratories, Indianapolis, IN) from 14 to 18 days post coitum and from neonates 1, 5, 10, and 15 days after birth ($n = 5$ per group). Pelvic limb muscle was collected from three 150-day-old normal ($Normal^{Mat}/Normal^{Pat}$) and three callipyge ($Normal^{Mat}/CLPG^{Pat}$) sheep.

2.4. RNA isolation and reverse-transcription quantitative PCR (RT-qPCR)

Total RNAs were isolated from C2C12 cells as well as mouse and sheep muscle using Trizol (Invitrogen, Carlsbad, CA), and used for cDNA synthesis with the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reverse-transcription quantitative PCR (RT-qPCR) primer sets (Suppl. Table 2) for analyzing gene expression were confirmed by PCR before conducting RT-qPCR. Measured Ct values were used to calculate the relative expression level of the target genes using the comparative $2^{-\Delta\Delta Ct}$ method [26]. Cyclophilin B was used as an internal control, and the mean Ct of EV on day 0 was used for calculating the fold changes. To analyze the expression patterns of alternative splicing forms of the *Dlk1* gene in mouse muscle tissues, semi-quantitative RT-PCR was performed with the *Dlk1* primer set mDLK1-4 E/F and mDLK-SP-R2, and the ribosomal protein, large, P0 (*Rplp0*) gene was amplified for reference by using the primer set Rplp0-F and Rplp0-R (Suppl. Table 1).

2.5. Immunoblotting

Protein preparation and immunobloting procedures were conducted as described in our previous report [27]. Anti-DLK (C-19; 1:20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-DLK (A-17; 1:1000, Santa Cruz Biotechnology), and anti-HA-tag (6E2; 1:3000, Cell Signaling Technology, Danvers, MA) antibodies were used as primary antibodies, and HRP-conjugated bovine anti-goat IgG (1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA) and horse anti-mouse IgG (1:5000, Cell Signaling Technology) antibodies were used as secondary antibodies.

2.6. Immunostaining and quantification of length, thickness, and fusion rate of myotubes

Immunostaining of cells was performed as described previously with modification [28]. Anti-myosin heavy chain antibody (H-300; 1:200, Santa Cruz Biotechnology) was used as a primary antibody, and FITC-conjugated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories) was used as a secondary antibody. The nuclei of cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were taken using a fluorescence microscope equipped with a QImaging Micropublisher 5.0 CCD camera (QImaging, Burnaby, BC, Canada). The length, thickness, and fusion rate of myotubes were measured with three randomly selected images by using the ImageJ program (NIH, Bethesda, MD). Fusion rate was determined by dividing the number of nuclei in myotubes by the total number of nuclei.

2.7. Statistical analysis

Results are presented as mean and standard error of means. Comparisons between the Dlk1 forms were performed by using the one-way analysis of variance in each day, followed by Tukey's honestly significant difference test for multiple comparisons at the significance level (alpha = 0.05).

3. Results

3.1. Dlk1 isoform expression and cleavage in C2C12 cells

Four stable C2C12 cell lines were generated by infecting the retroviruses encoding no gene (EV), the *Dlk1*-A for production of both membrane-bound and soluble Dlk1, the *Dlk1*-C2 for the membrane-bound form, or the 6 EGF form for soluble Dlk1 (Fig. 1A) [14]. The endogenous Dlk1 was barely detectable in EV-transfected cells, indicating very low levels of endogenous *Dlk1* expression in the C2C12 cell line [23]. Two bands corresponding to around 50 kDa in the lane for the Dlk1-A form resulted from glycosylated and non-glycosylated forms of Dlk1-A protein (Fig. 1B). Unexpectedly, the Dlk1-C2 form, that reportedly did not undergo cleavage in 3T3-L1 adipocytes, can be cleaved in the C2C12 cells (Fig. 1B). Cleavage of Dlk1-C2, which has an HA-tag at the C-terminal end (intracellular), was confirmed with two antibodies, anti-DLK C-terminal antibody (C-19) and anti-HA-tag antibody for the cell lysate, and an anti-DLK N-terminal antibody (A-17) for the medium. It was further confirmed in other cell types, 3T3-L1 and HEK-293 cells, and even the A form with deletion of the juxtamembrane domain [25] was also revealed to be cleaved (Suppl. Fig. 1). Detection of the cleaved C-terminal residues (about 12 kDa) in the cell lysates and secreted soluble Dlk1 in the media of both Dlk1-A and -C2 cells clearly demonstrates the cleavage of the two proteins. However, relative amounts of the cleaved C-terminal and secreted extracellular domain from the C2 form were less than those observed for the A form (Fig. 1B and Suppl. Fig. 1). These important findings led us to consider that Dlk1-A is an intermediate form producing both soluble and membrane-bound Dlk1, while the majority of C2 form may remain as membrane-bound Dlk1 during myogenic differentiation.

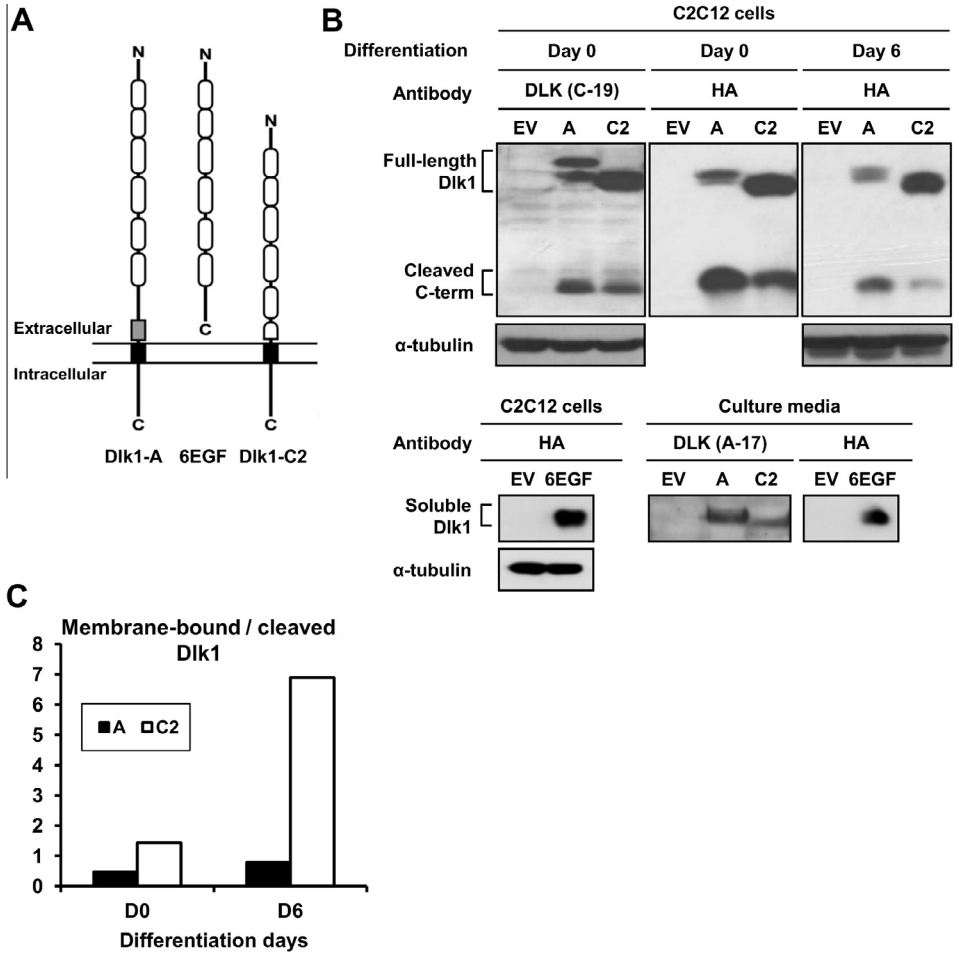

Fig. 1. Representative isoforms of Dlk1 and confirmation of overexpression and cleavage in C2C12 cell lines. (A) Representative isoforms of Dlk1. *Dlk1* has several alternative splicing forms and the cognate proteins are known to be proteolytically cleaved or not depending on the forms. The A form can be either bound to the membrane or cleaved to produce 6 EGF while the C2 form can only be anchored on the membrane as it has no protease-sensitive juxtamembrane domain (White oval: EGF domain, gray rectangular: protease-sensitive juxtamembrane domain, and black rectangular: transmembrane domain). (B) Expression and cleavage of Dlk1 in C2C12 cell lines. The Dlk1-A form was cleaved as expected, but also the C2 form was cleaved. The cleavage of Dlk1-A and -C2 isoforms was confirmed by using anti-Dlk1 (upper left) and anti-HA-tag (upper center and right) antibodies. The overexpression of 6 EGF in C2C12 cells was also confirmed by anti-HA tag antibody (lower left). The soluble Dlk1 derived from the A and C2 forms after cleavage, and 6 EGF, were detected in the medium using antibodies recognizing an amino-terminal proximal region of the protein or the HA tag, respectively (lower right).

3.2. Soluble Dlk1 decreases the length of myotubes, whereas the membrane-bound form increases the thickness of myotubes

Formation of myotubes was significantly affected by Dlk1 isoforms (Fig. 2A). The myotubes of the cells expressing the EV and C2-form cells were significantly longer than seen with expression of the A-form or 6 EGF (Fig. 2B; $P < 0.001$), indicating that the presence of soluble Dlk1 decreases myotube length. The thickness of myotubes was increased by C2-form (Fig. 2C; $P < 0.001$). An analysis of the average number of nuclei in the myotube revealed that the cells expressing the C2-form had significantly more nuclei than in the other groups, while the 6 EGF cells had the least number of nuclei (Fig. 2D). In addition, analysis of fusion rates revealed that the C2-form cells had the greatest fusion rate, followed by A-form with similar numbers in EV control and 6 EGF cells (Fig. 2E; $P < 0.001$). Overall, soluble Dlk1 inhibited myotube formation, while membrane-bound Dlk1 led to development of a hypertrophic phenotype.

3.3. Genes inducing myogenic differentiation are differently regulated by the Dlk1 isoforms

To analyze how Dlk1 isoforms affect myogenesis, relative expression of the genes related to myogenesis was measured (Fig. 3). *Pax7* was expressed approximately 2-fold higher before differentiation and 4-fold higher during differentiation in the 6 EGF cell than in the other groups (Fig. 3A). *Myf5* expression was 2-fold higher in the C2-form cells than in the 6 EGF cells before differentiation (Fig. 3B). *MyoD* expression was higher in the C2-form cells than in the 6 EGF cells at day 1 and greater in A- and C2-form cells than in EV and 6 EGF cells at day 4 (Fig. 3C). *Myog* increased continuously during differentiation, but decreased significantly only in 6 EGF cells at day 4 (Fig. 3D).

3.4. Genes regulated by membrane-bound Dlk1s to induce a hypertrophic phenotype and inhibition of differentiation by the soluble form

Expression of several genes related to muscle hypertrophy was analyzed to identify mediators of Dlk1. As shown in Fig. 4A, expression of myostatin (*Mstn*) increased dramatically about 30-fold in the EV cells at day 4 compared to day 0, while the other cells maintained *Mstn* expression at basal levels ($P < 0.05$). Follistatin (*Fst*) was down-regulated in 6 EGF cells compared to the other groups, whereas it was up-regulated in C2-form cells at day 4 (Fig. 4B; $P < 0.05$). Expression of Caveolin 3 (*Cav3*), an essential factor for myocyte fusion, was increased during differentiation in all groups,

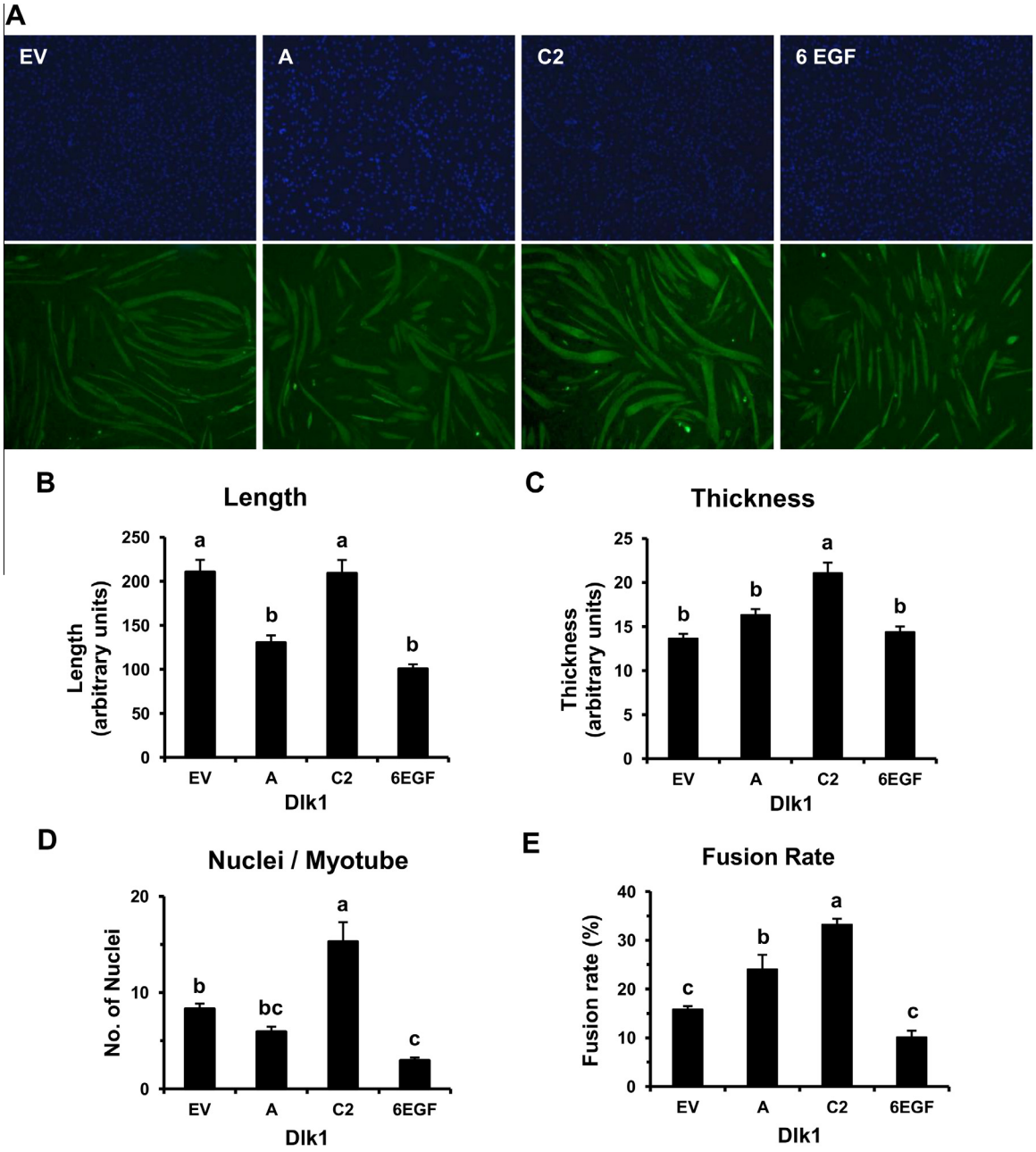

Fig. 2. Differentiation of C2C12 cells expressing different isoforms of Dlk1 and characterization of myotubes. (A) The C2C12 cells expressing different forms of Dlk1 or no Dlk1 (EV for empty vector not encoding Dlk1, A for A-form of Dlk1, C2 for C2-form of Dlk1, and 6 EGF for soluble form of Dlk1) were differentiated for 6 days. Cells were stained with DAPI (upper panels) and anti-myosin heavy chain antibody (lower panels; magnification = 35×). (B–E) The length and thickness of fibers, nuclei per myotube, and fusion rate were determined for each Dlk1 form. The unit of length is arbitrary based on the measured pixels (n = 3). The letters on the top of bars were determined by Tukey's HSD test (alpha = 0.05) and the bars with the same letter are not significantly different.

but was significantly higher in EV (1.5-fold) and C2-form (3-fold) than in 6 EGF cells at day 4 (Fig. 4C). *Cav3* was suppressed in the groups producing soluble Dlk1, but was not affected by membrane-bound Dlk1 as compared to the EV. The expression of *Bmp4*, an inhibitor of myocyte differentiation, was down-regulated by A- and C2 forms during day 0–2, but up-regulated by 6 EGF during day 1–2 (Fig. 5A). Expression of Noggin (*Nog*), an antagonist of BMP, was greater in the C2- and A-form cells than in the EV and 6 EGF cells before and during differentiation (Fig. 5B), indicating up-regulation of *Nog* by membrane-bound Dlk1. Lastly, the expression of genes involved in atrophy/dystrophy, including Atrogin1, muscle-specific RING finger protein 1 (*Murf1*), forkhead box O3 (*Foxo3*), and Dystrophin, was not significantly different among the groups (Fig. 5C–F), suggesting that signals related to muscle wasting may not be related to changes in myotube formation in response to Dlk1 isoforms.

3.5. The regulation of Dlk1 gene expression and cleavage of the protein is important for normal development of muscle in vivo

Analysis of alternative splicing patterns of *Dlk1* mRNA revealed that the primarily expressed *Dlk1* isoforms were A and C2 during muscle development in mice (Fig. 6A). The A form was expressed more abundantly than the C2 form throughout the time points.

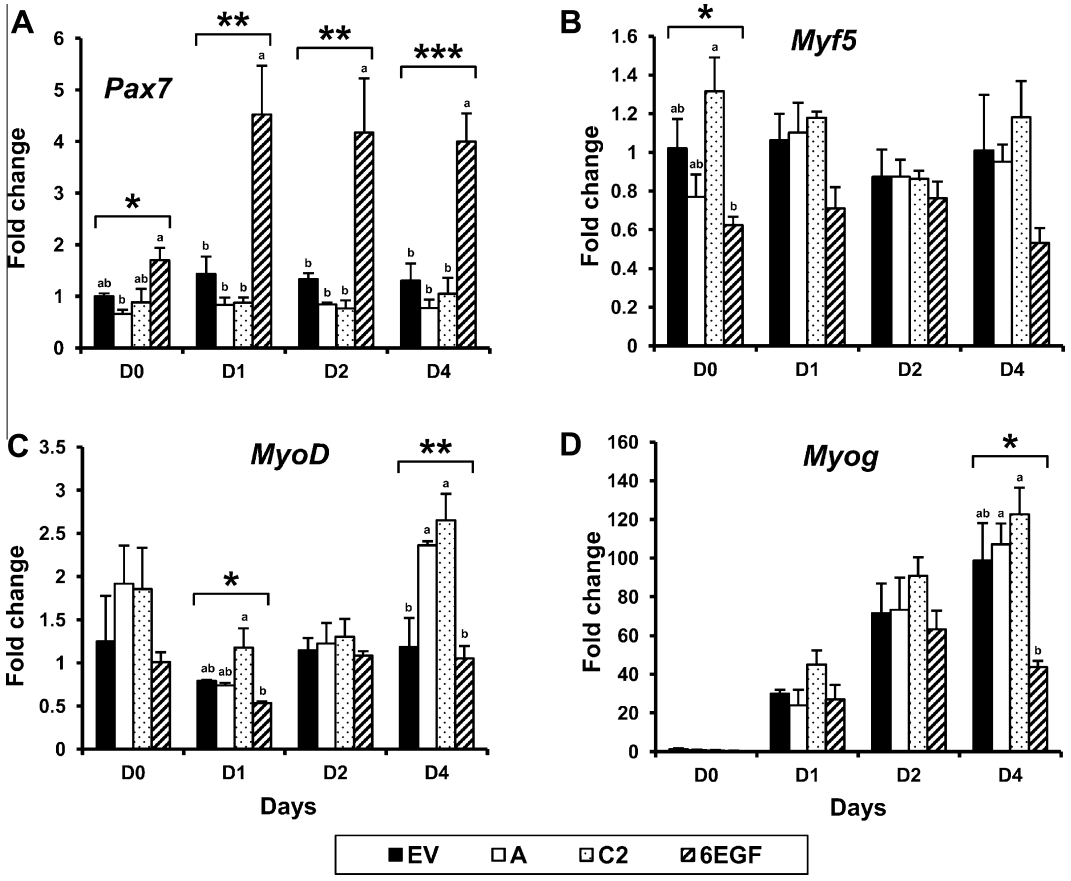

Fig. 3. The expression of myogenic genes. The expression of the myogenic factors *Pax7* (A), *Myf5* (B), *MyoD* (C), and *Myog* (D), were determined on day 0, 1, 2 and 4 of differentiation by using RT-qPCR ($n = 3$). *, **, and *** indicate significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The bars with the same letter are not significantly different within the day.

Both A and C2 forms were highly expressed from fetal ages up to day 5 after birth, but they were dramatically reduced after day 5. Immunobloting analysis (Fig. 6B) showed that three major bands of membrane-bound Dlk1 were as expected detected in mouse muscle, as reported in the previous study [20]. The level of total membrane-bound Dlk1 protein continuously decreased after birth ($P < 0.001$; Fig. 6B and C). Dlk1-A was a major form of membrane-bound Dlk1 at embryonic day 17; however, it decreased more rapidly than Dlk1-C2 and became a minor form at day 5 after birth (Fig. 6C). Cleavage of Dlk1 protein also decreased continuously after birth and was barely detectable at day 10 after birth ($P < 0.001$; Fig. 6B and D). The ratio of membrane-bound Dlk1 to cleaved Dlk1 was increased significantly on day 10 ($P < 0.001$; Fig. 6B and E). This indicates that the cleavage of Dlk1 protein is decreased, and the relative amount of membrane-bound Dlk1 is increased.

Abnormally high expression of *DLK1*-C2 isoform is responsible for muscle hypertrophy of callipyge sheep [11,19], which was also confirmed by about 30-fold greater expression level of *DLK1* in the muscle of callipyge sheep compared to normal sheep ($P < 0.05$; Fig. 6F). The expression patterns of several genes, including *MSTN*, *BMP4* and *PAX7*, were similar to the results obtained from the C2C12 cell overexpressing *Dlk1*-C2 (Fig. 6G). Overall, the expression pattern of downstream genes regulated by membrane-bound DLK1 *in vivo* was similar to that observed *in vitro*.

4. Discussion

Unlike the 3T3-L1 cells expressing endogenous *Dlk1*, the C2C12 cells having undetectable levels of Dlk1, as reported by another group [23], could serve as an excellent *in vitro* system to study the function of exogenously expressed Dlk1 isoforms. Although Dlk1-C2 form was known not to be cleaved [14], our immunoblotting analysis using several different antibodies clearly demonstrated that Dlk1-C2 is cleaved and generates a soluble Dlk1 in C2C12, 3T3-L1, and HEK-293 cells. Moreover, the Dlk1-dA, Dlk1-A without the juxtamembrane domain, could also be cleaved at a detectable level and generate the soluble form (Suppl. Fig. 1). These data suggest that the Dlk1 isoforms without the juxtamembrane domain can be noticeably cleaved. However, it was not determined which protease, including TACE, could cleave the Dlk1 isofoms without the juxtamembrane domain. Due to the fact that the majority of the C2 form remained intact, it was considered a major membrane-bound Dlk1 in this study, with the A form representing an intermediate form.

The most important finding in this study is that membrane-bound Dlk1 promotes hypertrophic myotube formation, whereas soluble Dlk1 inhibits myotube formation. The thicker myotubes with greater fusion rates generated by membrane-bound Dlk1 may be mediated by up-regulation of myogenic transcription factors (*Myf5*, *MyoD*, and *Myog*), genes promoting hypertrophy (*Fst* and *Nog*), and down-regulation of anti-myogenic genes (*Mstn* and *Bmp4*). In addition, the expression pattern of the genes *Mstn*, *Bmp4*, and *Pax7*, in the hypertrophic muscle of callipyge sheep, caused by an abnormally high expression of the *DLK1*-C2 as a major form [11], was very similar to that of C2C12 cells expressing *Dlk1*-C2. Considering the similar phenotype of muscle and expression patterns of genes between callipyge sheep and C2C12 cells expressing *Dlk1*-C2, the membrane-bound form is likely to induce hypertrophy in muscle. This is

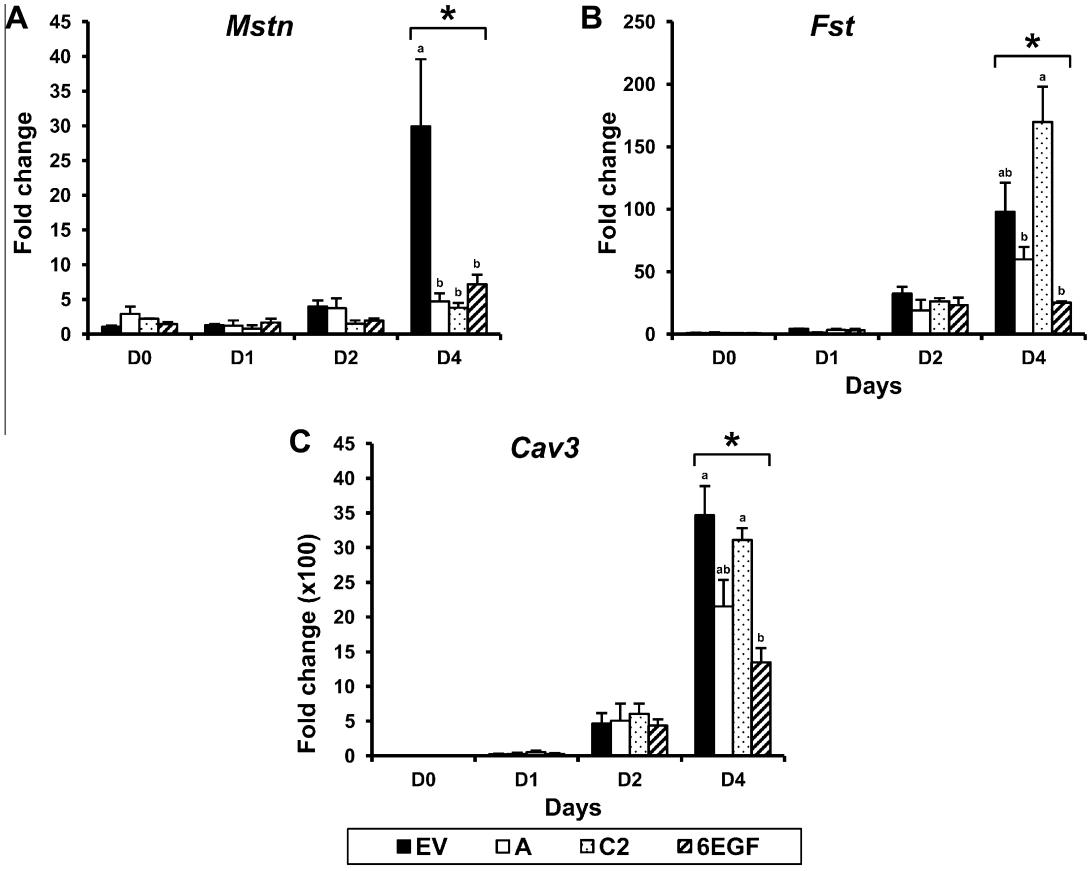

Fig. 4. The expression of genes related to muscle hypertrophy. The expression of genes known to inhibit or induce muscle hypertrophy, *Mstn* (A), *Fst* (B), and *Cav3* (C), were determined on days 0, 1, 2 and 4 of differentiation by using RT-qPCR ($n = 3$). * and ** indicate significance levels of $P < 0.05$ and $P < 0.01$, respectively. The bars with the same letter are not significantly different within the day.

further supported by the studies showing a hypertrophic muscle in transgenic mice overexpressing sheep *DLK1*-C2 in the skeletal muscle [11].

The inhibitory roles of soluble Dlk1 in myogenesis was accompanied by the down-regulation of myogenic transcription factors (*Myf5*, *MyoD*, and *Myog*) and up-regulation of genes (*Pax7*, and *Bmp4*) inhibiting the differentiation and sparing the myoblasts during differentiation. Especially increased *Bmp4* expression and reduced expression of *Nog*, an antagonist of BMP, by soluble Dlk1 could contribute to inhibiting myogenic differentiation [9], suggesting *Bmp4* might be an effective mediator of soluble Dlk1. The down-regulation of *Cav3*, an essential factor for myocyte fusion, by soluble Dlk1, could be associated with short myotubes. Taken together, dramatically up-regulated *Pax7* and *Bmp4* and down-regulation of *Myf5* and *MyoD* indicate that soluble Dlk1 could halt the myogenic process at early stages, possibly leading to maintaining more myogenic progenitor cells. Soluble Dlk1 seems to have a general inhibitory effect on cellular differentiation, including adipocyte differentiation, the differentiation of myocytes into adipocytes, mesenchymal stem cells into adipocytes and osteoblasts, and chondrogenic cell differentiation [14,25,29–32].

During perinatal ages in mice, total amounts of Dlk1 were gradually reduced, but the cleavage of Dlk1 protein was dramatically decreased, resulting in an increased ratio of membrane-bound to soluble Dlk1 in postnatal muscle tissues. Both membrane-bound and soluble Dlk1 abundantly found during the embryonic ages may have a role in balancing between differentiation and maintenance of myogenic precursor cells, respectively. However, increasing ratios of membrane-bound to soluble form after birth may promote differentiation of myogenic cells and maturation of myofibers, which is associated with the acquisition of physical ability of muscle. The switching of Dlk1 forms *in vivo* may occur through two different mechanisms; regulation of *Dlk1* isoform expression and protease shedding of Dlk1 proteins. Because mice, cattle, sheep, and pigs have two or more alternative splicing isoforms of *Dlk1* [11,13,19], they would use both mechanisms to regulate ratios of membrane-bound to soluble DLK1. However, in humans and birds expressing only a full-length *Dlk1* [16,18], the regulation of DLK1 shedding may be a more important mechanism for switching DLK1 forms. Taken together, temporal expression of *Dlk1* during embryonic and neonatal ages should be tightly regulated for normal muscle development and maturation.

Abnormal muscle development in maternal or paternal uniparental disomy (m/pUPD) chromosome 12 is known to be caused by dysregulation of *Dlk1* expression [33,34], suggesting the importance of *Dlk1* regulation in myogenesis. The absence of *Dlk1* expression in mouse mUPD12 fetus resulted in premature muscle formation and fewer numbers of muscle fibers, leading to hypotonia in neonates [35,36]. Considering the given negative influence of soluble Dlk1 on myogenic differentiation and positive influence of membrane-bound Dlk1 on myofiber formation, it is possible that premature muscle in the mUPD12 fetus could be caused by the absence of soluble Dlk1 and fewer numbers of myofibers by the absence of membrane-bound Dlk1. In support of this explanation,

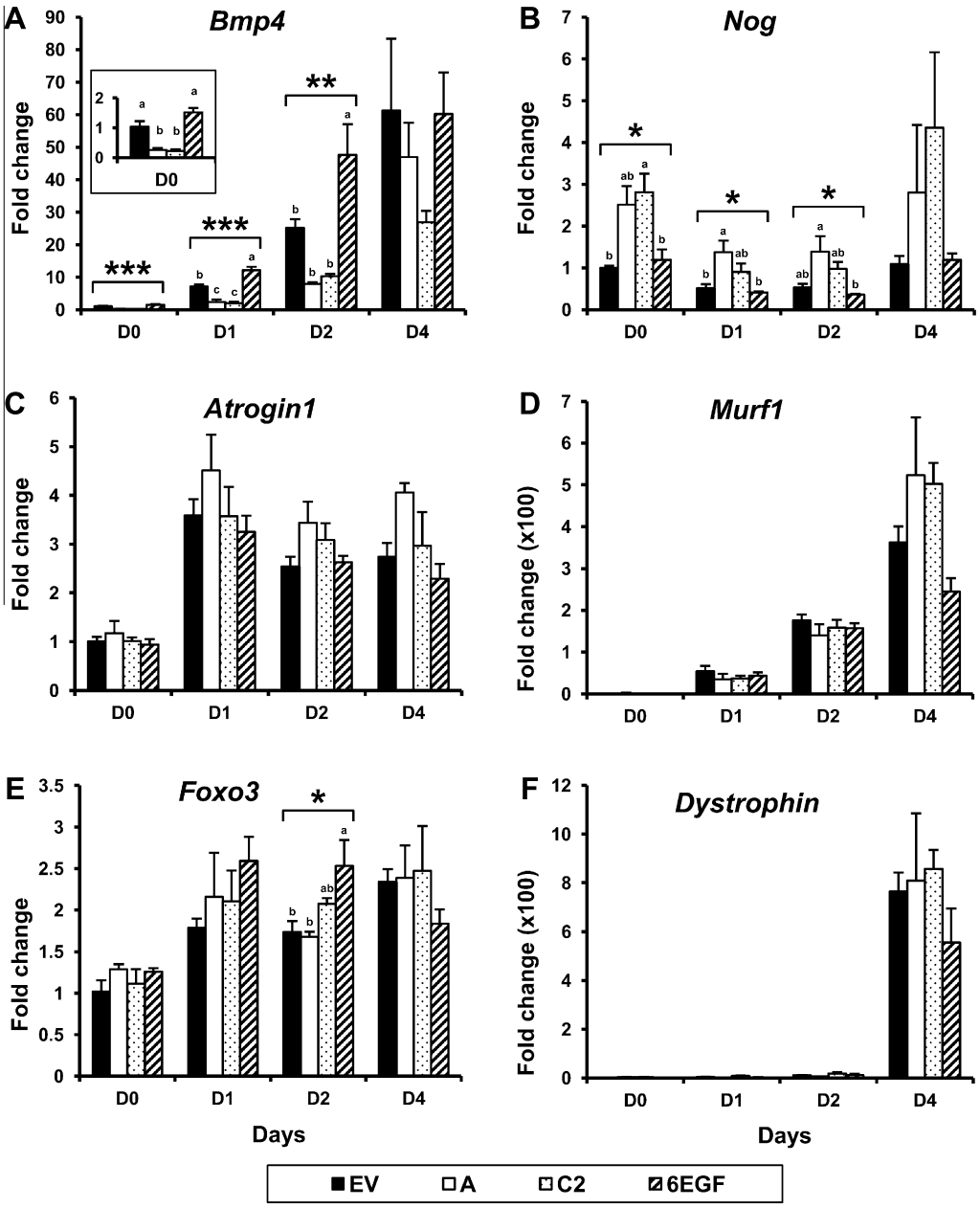

Fig. 5. The expression of genes related to inhibition of myogenic differentiation and muscle wasting. The expression of *Bmp4* (A), which is known to inhibit myogenic differentiation, and its antagonist (*Nog*; B), as well as expression of *Atrogin1* (C), *Murf-1* (D), *Foxo3* (E), and Dystrophin (F), which are known to be related to muscle atrophy/dystrophy, were determined on days 0, 1, 2 and 4 of differentiation by using RT-qPCR ($n = 3$). *, **, and *** indicate significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The bars with the same letter are not significantly different within the day.

the pUPD12 mice expressing a double dosage of *Dlk1* showed delayed muscle maturation and higher fiber numbers [36], which are likely caused by abnormally high amounts of soluble and membrane-bound Dlk1, respectively. Our findings from the current studies provide a basis to explain distinct phenotypic differences in muscle development of mUPD12 and pUPD12 fetuses.

In conclusion, different forms of Dlk1 have distinctive effects on myogenesis. Membrane-bound Dlk1 enhances myotube formation and hypertrophy, while soluble Dlk1 inhibits myocyte differentiation and myotube formation, demonstrating that the regulation of Dlk1 isoforms during myogenesis is critical for normal muscle development.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.027.

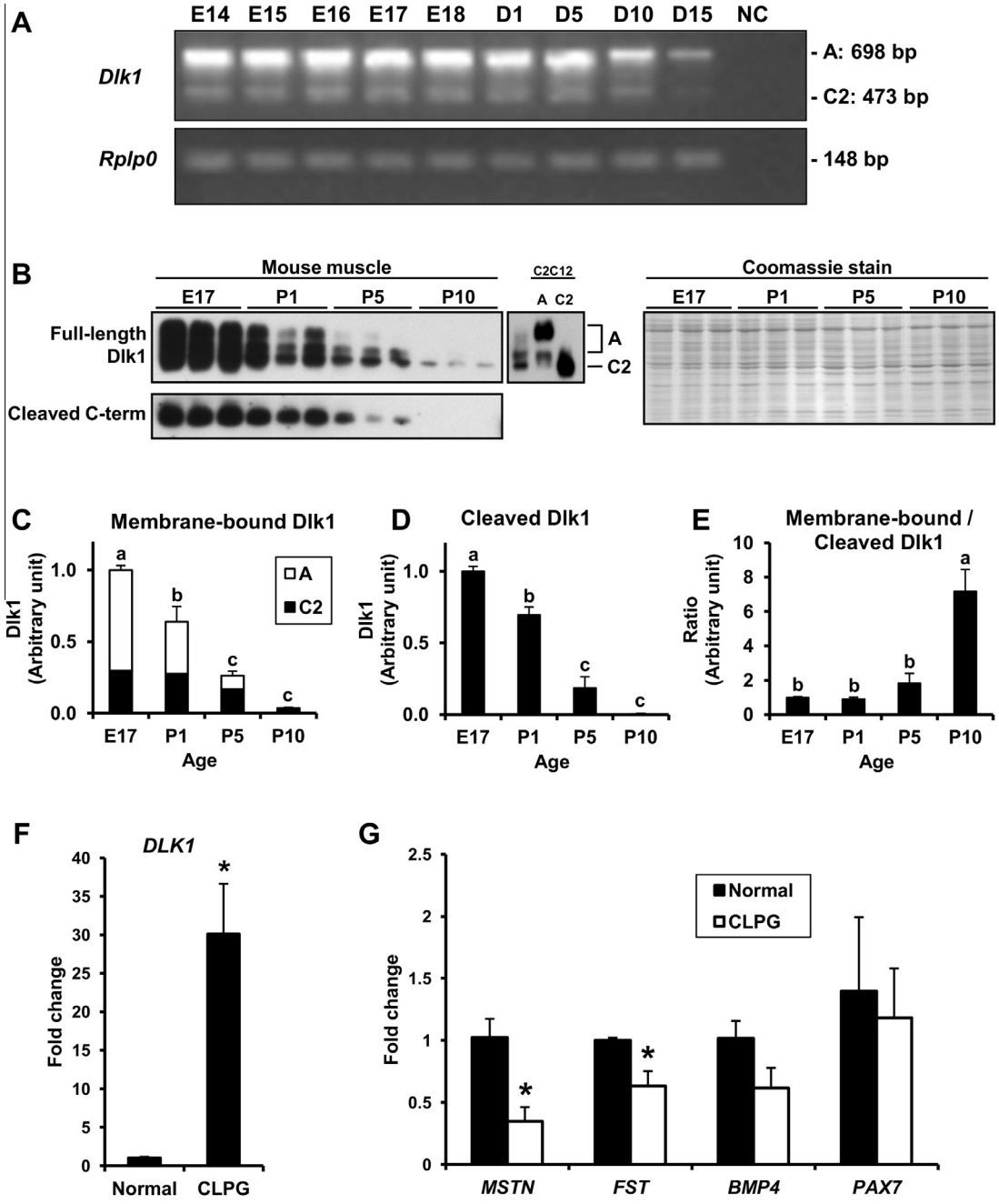

Fig. 6. *Dlk1* isoform expression and proteolytic cleavage in muscle of mice and callipyge sheep. (A) The expression of the *Dlk1* gene during mouse muscle development from 14 to 18 days post coitum and 1, 5, 10, and 15 days after birth. Mainly *Dlk1*-A and C2 isoforms were amplified by PCR in mouse muscle. However, the expression levels were decreased 10 days after birth. The *Rplp0* gene was used for reference. (B) The expression level and cleavage of Dlk1 protein during mouse muscle development from 17 days post coitum and 1, 5, and 10 days after birth. Total protein concentrations were determined by Coomassie staining. (C–E) Quantification of Dlk1 protein expression from panel B. Each Dlk1 band was normalized by using the Coomassie staining result. Among the membrane-bound Dlk1 forms, the A and C2 forms were distinguishable by using C2C12 cells overexpressing the A and C2 form as positive controls. The ratio of membrane-bound to cleaved Dlk1 was determined. The bars with the same letter are not significantly different. (F and G) The expression of *Dlk1* and important genes related to induction of muscle hypertrophy or inhibition of differentiation were analyzed in the adult pelvic limb muscle of normal sheep ($n = 3$) vs. callipyge sheep ($n = 3$) at the age of 150 days. Asterisk indicates a significance level of $P < 0.05$.

References

[1] Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51, 987–1000.

[2] Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H.H. (1989) A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. 8, 701–709.

[3] Edmondson, D.G. and Olson, E.N. (1989) A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. 3, 628–640.

[4] Rhodes, S.J. and Konieczny, S.F. (1989) Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. 3, 2050–2061.

[5] Olguin, H.C. and Olwin, B.B. (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev. Biol. 275, 375–388.

[6] Kopan, R., Nye, J.S. and Weintraub, H. (1994) The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development 120, 2385–2396.

[7] Conboy, I.M. and Rando, T.A. (2002) The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev. Cell 3, 397–409.

[8] Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C.F. and Lendahl, U. (2003) Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130, 6089–6099.
[9] Ono, Y., Calhabeu, F., Morgan, J.E., Katagiri, T., Amthor, H. and Zammit, P.S. (2011) BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells. Cell Death Differ. 18, 222–234.
[10] Charlier, C., Segers, K., Karim, L., Shay, T., Gyapay, G., Cockett, N. and Georges, M. (2001) The callipyge mutation enhances the expression of coregulated imprinted genes in cis without affecting their imprinting status. Nat. Genet. 27, 367–369.
[11] Davis, E. et al. (2004) Ectopic expression of DLK1 protein in skeletal muscle of padumnal heterozygotes causes the callipyge phenotype. Curr. Biol.: CB 14, 1858–1862.
[12] White, J.D., Vuocolo, T., McDonagh, M., Grounds, M.D., Harper, G.S., Cockett, N.E. and Tellam, R. (2008) Analysis of the callipyge phenotype through skeletal muscle development; association of Dlk1 with muscle precursor cells. Differentiation Res. Biol. Div. 76, 283–298.
[13] Smas, C.M., Green, D. and Sul, H.S. (1994) Structural characterization and alternate splicing of the gene encoding the preadipocyte EGF-like protein pref-1. Biochemistry 33, 9257–9265.
[14] Smas, C.M., Chen, L. and Sul, H.S. (1997) Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. Mol. Cell. Biol. 17, 977–988.
[15] Wang, Y. and Sul, H.S. (2006) Ectodomain shedding of preadipocyte factor 1 (Pref-1) by tumor necrosis factor alpha converting enzyme (TACE) and inhibition of adipocyte differentiation. Mol. Cell. Biol. 26, 5421–5435.
[16] Deiuliis, J.A., Li, B., Lyvers-Peffer, P.A., Moeller, S.J. and Lee, K. (2006) Alternative splicing of delta-like 1 homolog (DLK1) in the pig and human. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 145, 50–59.
[17] Floridon, C., Jensen, C.H., Thorsen, P., Nielsen, O., Sunde, L., Westergaard, J.G., Thomsen, S.G. and Teisner, B. (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differentiation; Res. Biol. Div. 66, 49–59.
[18] Shin, S., Han, J.Y. and Lee, K. (2010) Cloning of avian Delta-like 1 homolog gene: the biallelic expression of Delta-like 1 homolog in avian species. Poult. Sci. 89, 948–955.
[19] Vuocolo, T., Pearson, R., Campbell, P. and Tellam, R.L. (2003) Differential expression of Dlk-1 in bovine adipose tissue depots. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 134, 315–333.
[20] Moon, Y.S., Smas, C.M., Lee, K., Villena, J.A., Kim, K.H., Yun, E.J. and Sul, H.S. (2002) Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol. Cell. Biol. 22, 5585–5592.
[21] Lee, K., Villena, J.A., Moon, Y.S., Kim, K.H., Lee, S., Kang, C. and Sul, H.S. (2003) Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). J. Clin. Investig. 111, 453–461.
[22] Andersen, D.C., Petersson, S.J., Jorgensen, L.H., Bollen, P., Jensen, P.B., Teisner, B., Schroeder, H.D. and Jensen, C.H. (2009) Characterization of DLK1+ cells emerging during skeletal muscle remodeling in response to myositis, myopathies, and acute injury. Stem Cells 27, 898–908.
[23] Waddell, J.N. et al. (2010) Dlk1 is necessary for proper skeletal muscle development and regeneration. PLoS ONE 5, e15055.
[24] Andersen, D.C., Laborda, J., Baladron, V., Kassem, M., Sheikh, S.P. and Jensen, C.H. (2013) Dual role of delta-like 1 homolog (DLK1) in skeletal muscle development and adult muscle regeneration. Development 140, 3743–3753.
[25] Mei, B., Zhao, L., Chen, L. and Sul, H.S. (2002) Only the large soluble form of preadipocyte factor-1 (Pref-1), but not the small soluble and membrane forms, inhibits adipocyte differentiation: role of alternative splicing. Biochem. J. 364, 137–144.
[26] Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25, 402–408.
[27] Lee, K., Li, B., Xi, X., Suh, Y. and Martin, R.J. (2005) Role of neuronal energy status in the regulation of adenosine 5'-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 146, 3–10.
[28] Li, B., Shin, J. and Lee, K. (2009) Interferon-stimulated gene ISG12b1 inhibits adipogenic differentiation and mitochondrial biogenesis in 3T3-L1 cells. Endocrinology 150, 1217–1224.
[29] Smas, C.M. and Sul, H.S. (1993) Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 73, 725–734.
[30] Abdallah, B.M., Jensen, C.H., Gutierrez, G., Leslie, R.G., Jensen, T.G. and Kassem, M. (2004) Regulation of human skeletal stem cells differentiation by Dlk1/Pref-1. J. Bone Miner. Res. 19, 841–852.
[31] Chen, L. et al. (2011) Delta-like 1/fetal antigen-1 (Dlk1/FA1) is a novel regulator of chondrogenic cell differentiation via inhibition of the Akt kinase-dependent pathway. J. Biol. Chem. 286, 32140–32149.
[32] Abdallah, B.M., Beck-Nielsen, H. and Gaster, M. (2013) FA1 induces pro-inflammatory and anti-adipogenic pathways/markers in human myotubes established from lean, obese, and type 2 diabetic subjects but not insulin resistance. Front. Endocrinol. 4, 45.
[33] Murphy, S.K., Wylie, A.A., Coveler, K.J., Cotter, P.D., Papenhausen, P.R., Sutton, V.R., Shaffer, L.G. and Jirtle, R.L. (2003) Epigenetic detection of human chromosome 14 uniparental disomy. Hum. Mutat. 22, 92–97.
[34] Schmidt, J.V., Matteson, P.G., Jones, B.K., Guan, X.J. and Tilghman, S.M. (2000) The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes Dev. 14, 1997–2002.
[35] Berends, M.J., Hordijk, R., Scheffer, H., Oosterwijk, J.C., Halley, D.J. and Sorgedrager, N. (1999) Two cases of maternal uniparental disomy 14 with a phenotype overlapping with the Prader–Willi phenotype. Am. J. Med. Genet. 84, 76–79.
[36] Georgiades, P., Watkins, M., Surani, M.A. and Ferguson-Smith, A.C. (2000) Parental origin-specific developmental defects in mice with uniparental disomy for chromosome 12. Development 127, 4719–4728.