Age-dependent variations in the expression of myosin isoforms and myogenic factors during the involution of the proximal sesamoidean ligament of sheep

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ABSTRACT

In ungulates the stability of the fetlock joint is dependent on several muscles, which are exposed to high stress and strain. Among those muscles, the proximal sesamoidean ligament or PSL (also known as the suspensory ligament or Ruini's elasto-tendinous organ) is organized at birth in layers of muscle fibres alternated with abundant tendinous tissue that, during the postnatal development, becomes the predominant tissue.

In this study we analysed the PSL of the sheep at the age of 1, 30 and 180 days and determined the expression of several genes which either (a) are markers of muscle fibre growth and maturation, or (b) play a role as signal molecules. We observed an accelerated maturation, as indicated by the transition of MyHC isoform expression towards the slow isoforms and a reduced regenerative potential indicated by the low Pax7 expression and the altered Wnt signalling. We also found a specific myogenic expression pattern of MyoD, Myf5 and Myogenin in the developing PSL and high mRNA levels of specific fibrogenic factors, as TGF-β1, that, undoubtedly, stimulate the growth of connective tissue. Our observations confirmed, at molecular level, the peculiarity of the fast involution observed in PSL a muscle that undergoes a very specific active differentiation process during early development, which implies myofibres involution and their replacement with connective tissue.

1. Introduction

Diversity and specialization are essential features of skeletal muscles. The diversity represents the basis of their ability to accomplish a variety of tasks: from posture to locomotion, from breathing to phonation. Muscle diversity implies a structural organization, which involves specific components, in the first place the muscle fibres or myofibres and also other components as connective tissue, vessels and nerves. The role of connective tissue inside skeletal muscles is particularly important as layers of connective tissue surround the whole muscle (epimysium) and penetrate among fibre bundles (perimysium) to reach and make contact with individual fibres (endomysium). The contribution of the connective tissue is not only structural, i.e. to organize the architecture of the muscle, but also functional as connective tissue is responsible of force transmission. The diversity of skeletal muscles is the result of interactions between genetically predetermined programs of development and environmental factors. A notable example in this respect is a particular muscle especially important in ungulates, the proximal sesamoidean ligament, which was analysed in its morphological transformation in a previous paper of our group (Mascarello and Rowlerson, 1995) and a recent one (Melotti et al., 2019). Specifically, the proximal sesamoidean ligament (PSL; also known as the suspensory ligament or Ruini's elasto-tendinous organ) is a homologue of the medial (3rd) interosseus muscle in horses and of the 3rd and 4th interosseus muscles in ruminants (Mascarello and Rowlerson, 1995). During the development of this muscle, muscle fibres grow in layers alternated with layers of abundant connective tissue. The muscle component of the PSL is always more abundant in forelimbs compared with hindlimbs in all species and at all ages (Ottolenghi, 1931; Ottolenghi, 1932; Godina, 1935; Aureli, 1962). This has been attributed to the different roles played by the forelimbs and hindlimbs (mostly, postural and propulsive, respectively) in quadruped

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Table 1
Used primers.

Gene	Accession number	Primer sequences (5′– > 3′)	Size (pb)
MyHC-1	XM_012122420.2	F: GTGCAAAGAAAGGTGGCAAGA R: GTGCTCCTCAGGTTGGTCATC	100
MyHC-2A	XM_012122422.2	F: AGGACCCCCTGAACGATACC R: CCCTCTGGATTCAGCAGTTGGA	101
MyHC-2B	XM_012122419.2	F: GGCTGGCTGGACAAGAACAA R: GACCTCCAGCGAAGAGGAAA	100
MyHC-2X	XM_012129251.1	F: CTTTGCCAACTATGCTGGGTTT R: CTCCCTGTGCAGAGCTGACA	100
MyHC EMB	NM_001101835.1	F: AGAAGAACAAGGACCCCCTGAA R: TCCGCGGTGGTGAAGGT	100
MyHC Neo	XM_012122417.2	F: CAAGGACCCCCTGAATGACA R: GTCAGCTTCAGCACTAGCATATGTG	100
MyHC-EO	XM_015105526.1	F: ACAAGGACCCCCTGAATGAGA F: CCTGCTTCTGCACCAGCATA	100
Pax7	XM_015109012.1	F: AGGACGGCGAGAAGAAAGC R: AGGGAGGTCGGGTTCTGACT	104
MyoD	NM_001009390.1	F: GCCGCTTGAGCAAAGTCAA R: TGCGTTGCGCAGGATCT	101
Myf5	XM_012141453.2	F: TGCCACGGATAAAAGCTCCTT R: CTGGATCCTGGAGAGGCAATC	101
MYOG	NM_001174109.1	F: GTAAGGTGTGCAAGCGGAAGT R: GCCTCGAAGGCTTCATTCAC	100
Wnt1	XM_012161835.2	F: TGCAGCGACAACATCGACTT R: CCCGCCTCATTGTTGTGAA	110
Wnt4	XM_012115458.2	F: CTCGGATAACATCGCCTATGG R: GGCCTCATTGTTGTGGAGGTT	115
IGF-1	NM_001009774.3	F: CCAGACAGGAATCGTGGATG R: ACTTGGCGGGCTTGAGAG	89
MSTN	NM_001009428.2	F: TCCACTCCGGGAACTGATTG R: GACCGTTTCCGTCGTAACGT	100
VEGF	NM_001025110.1	F: GCTCTCTTGGGTGCATTGGA R: TGCAGCCTGGGACCACTT	70
TGF-β1	XM_024977951.1	F: GAACTGCTGTGTTCGTCAGC R: GGTTGTGCTGGTTGTACAGG	170

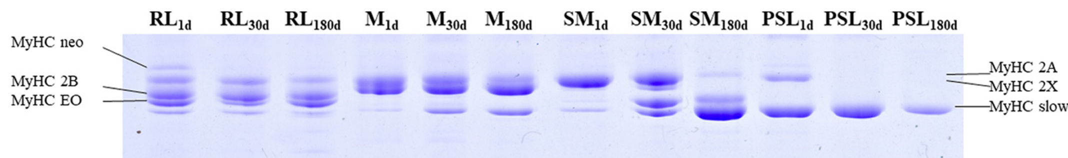


Fig. 1. SDS-PAGE gel of MyHC isoforms expression at different ages. Samples of rectus lateralis extraocular muscle (RL), masseter muscle (M), semimembranosus muscle (SM) and proximal sesamoidean ligament (PSL) were collected at 1, 30 and 180 days after birth (1d, 30d and 180d respectively) and analysed on a 8% acrylamide gel (loaded 8 mg/lane), according to Talmadge and Roy protocol (Talmadge and Roy, 1993), in order to separate different Myosin Heavy Chains (MyHCs) isoforms (slow: MyHC-1; fast: MyHC-2A, MyHC-2X and MyHC-2B; neonatal: MyHC neo; extraocular: MyHC EO) expressed in the muscle. The gel was stained with Coomassie blue.

locomotion.

The aim of this study was to further develop our previous results, increasing molecular data and following more closely the maturational transitions of Myosin Heavy Chain (MyHC) isoforms expression which are best markers of fibre type (Schiaffino and Reggiani, 2011). In this frame, we aim to answer the still open questions whether a specific myogenic program drives the accelerated maturation and involution of PSL and whether some specific signalling factors present in the niche can inhibit the activation of satellite cells or stimulate the growth of connective tissue. In our previous study (Mascarello and Rowlerson, 1995), we took as reference for comparison the maturation of two other sheep muscles, namely the masseter and the semimembranosus. In the present study, we have added as a third reference an extraocular muscle, the rectus lateralis, assuming that these muscles can undergo a continuous remodeling (Schoser and Pongratz, 2006; Patruno et al., 2008).

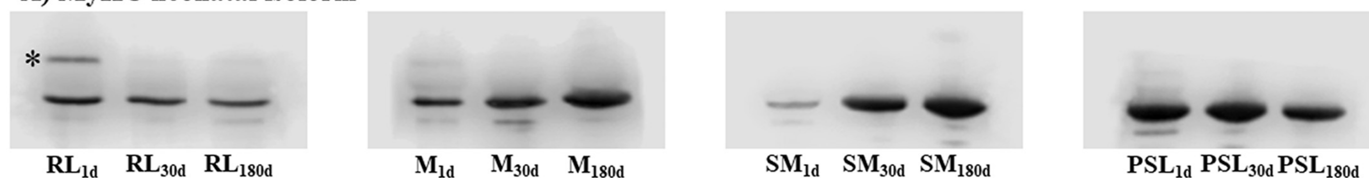
2. Materials and methods

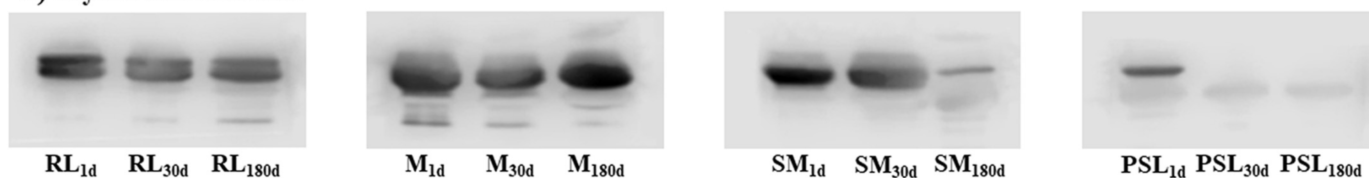
2.1. Sample preparation

Samples were collected at the local slaughter house from proximal sesamoidean ligament (PSL), masseter (M), semimembranosus (SM) and the extraocular muscles rectus lateralis (RL) of 10 sheep at the following ages: 1 day (1d), 30 days (30d) and 180 days (180d) after birth. Each muscle sample was divided in two parts: the first part was immersed in Laemmli solution for SDS-PAGE and western blot analysis and the second part was immersed in RNA*later* solution for Real Time PCR analysis. The national and institutional guidelines for the care and use of animals were followed and all procedures were approved by the local Institutional Animal Care and Use Committee.

2.2. SDS-PAGE and Western blotting

For the SDS-PAGE, samples were solubilized in Laemmli solution (62.5mMTris, pH 6.8, 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, with 0.1% E-64 and 0.1% leupeptin as antiproteolytic factors)





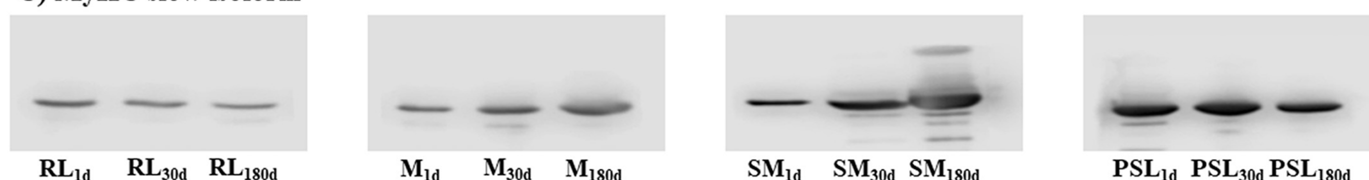


Fig. 2. Western blot analysis of MyHC isoforms expression at different ages. Samples of rectus lateralis extraocular muscle (RL), masseter muscle (M), semimembranosus muscle (SM) and proximal sesamoidean ligament (PSL) at different ages (1, 30 and 180 days) were loaded on a 8% acrylamide gel according to Talmadge and Roy (1993). After electrophoresis and transfer, nitrocellulose was stained with the antibodies BA-D5 against MyHC-1 (slow), SC-71 against the fast isoforms MyHC-2A & MyHC 2× and 2E9 against MyHC neonatal. The latter antibody shows a cross-reactivity with MyHC-1 (slow). Slow MyHC was expressed in all samples at all ages and in PSL was the only isoform expressed at 30d and 180d. Neonatal* MyHC was expressed at 1 d in RL. Fast MyHC isoforms were present in RL, M and SM, while in PSL only at 1d a band corresponding to MyHC-2A was present.

(Laemmli, 1970) and stored at −80 °C until required. After heating for 5 min at 80 °C, the protein suspension was loaded onto polyacrylamide gels. The separation of MyHC isoforms was achieved on 8% polyacrylamide slab gels with a protocol derived from Talmadge and Roy (1993). Slabs 18 cm wide, 18 cm high and 1 mm thick were used. Electrophoresis was run at 4 °C for 40 h, at 70 V for 1 h and 140 V for the remaining time. After the run, gels were stained with Coomassie blue to identify protein bands.

After the SDS-PAGE, gel bands were blotted onto a nitrocellulose membrane in order to perform western blot (WB) analysis for the identification of the different MyHC isoforms. After blotting, the membrane was stained with red Ponceau to verify the protein transfer. The membrane was then incubated with primary antibodies for the detection of the protein of interest overnight at 4 °C. Afterwards, the membrane was repeatedly washed and then incubated with the secondary antibody. The primary antibodies used were against MyHC-1 (BA-D5), MyHC-2A and MyHC 2× (SC-71) and MyHC neonatal (2E9); all these antibodies were obtained at the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA, USA).

MyHC isoforms were identified based on the migration rate and the antibody reactivity. As previously documented in ovine muscles (Hemmings et al., 2009), in antelopes (Kohn, 2014) and in other animal species (Schiaffino and Reggiani, 2011), the fastest migrating band corresponds to MyHC-1 (or slow) as confirmed by reactivity with BA-D5 antibody (see Schiaffino et al., 1989). The slowest migrating band corresponds to developmental isoforms as shown by the presence restricted to 1 day old samples and reactivity with 2E9 antibody (see Bandman, 1985). Several fast isoforms migrate between these two extremes: three are easily detectable in Rectus Lateralis and correspond from the slowest to the fastest to MyHC 2A, 2X and extraocular. As discussed by Kohn (2014) the relative migration rates of MyHC 2A and 2X change in relation to species and, in ungulates, MyHC 2X precedes MyHC 2A. The antibody SC-71 initially created to recognize MyHC 2A (Schiaffino et al., 1989), reacts also with MyHC 2X, although with a lower affinity (see Smerdu and Soukup, 2008).

2.3. RNA preparation and Real Time PCR

Samples collected for gene expression analysis were cut into small pieces, immediately immersed in RNA*later* solution (Ambion, Austin, TX, USA) and stored at −20 °C until required. Total RNA was isolated from samples of skeletal muscles using TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA). Total RNA (2 µg) was retrotranscribed using Superscript™ II Reverse Transcriptase protocol (Invitrogen, Carlsbad, CA, USA) and a mixture of random hexamers were used as primers in order to synthetize the first-strand cDNA. Possible trace of genomic DNA was removed using the DNase I treatment (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The obtained cDNAs were used as template for Real Time PCR.

The relative expression of genes involved in myogenesis (MyoD, Myf5 and Myogenin), Pax7 and myosin heavy chain isoforms (MyHC) was assessed by Real Time PCR. Moreover, several signalling factors, which might potentially contribute to stimulation or inhibition of muscle cell regeneration and fibrosis (Wnt1, Wnt4, IGF-1, Myostatin, VEGF and TGF-β1), were studied. Real Time PCR quantification of target and housekeeping genes was performed using the SYBR Green method with an Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described (Sacchetto et al., 2009). The calculation of the relative mRNA expression of target genes was obtained using the comparative threshold cycle (Ct) method using the Applied Biosystems 7500 SDS software. An endogenous control gene (18S) was used to normalize the quantification of target genes. Ct is the fractional cycle number at which the fluorescence passes the set threshold.

Relative quantification (RQ), or fold of change, was quantified as follows:

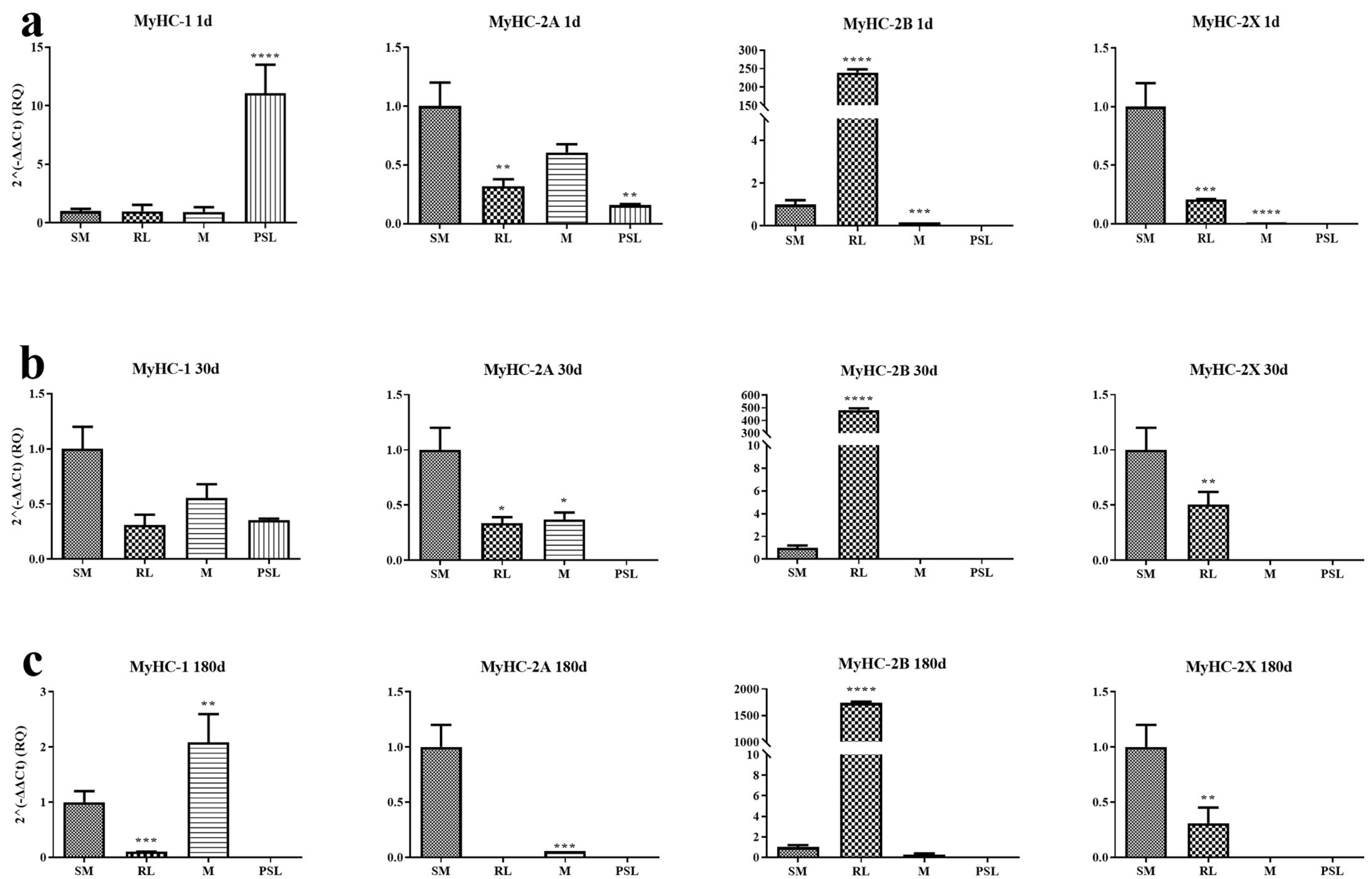


Fig. 3. MyHC isoforms gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of MyHC slow (MyHC-1) and fast isoforms (MyHC-2A, MyHC-2B and MyHC-2X) in the the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM, detected by Real Time PCR. SM was used as calibrator sample. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$.

$\Delta Ct = Ct_{target} - Ct_{reference}$

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$

$Fold\ of\ change = 2^{-\Delta\Delta Ct}$

In addition, melting curves were examined as well to confirm the specific amplification of the cDNA target and the absence of non-specific products. SM was used as the calibrator sample for the Real Time PCR relative quantification. Sheep specific oligonucleotide primers are listed in Table 1.

2.4. Statistical analysis

All data are expressed as mean + SEM. Statistical analysis were performed with the one-way ANOVA test followed by the post-hoc Tukey test to compare the data of the different experimental groups. The accepted level of significance was set at $p \leq .05$. All statistical analyses were performed with GraphPad PRISM version 5.00 (GraphPad software, La Jolla, CA, USA).

3. Results

3.1. Expression of myosin heavy chain isoforms

As a first step, in order to follow the transition in fibre type, which occurs during muscle development and maturation, we studied the expression of myosin isoforms, which are widely accepted markers of fibre types (Mascarello and Rowlerson, 1995). The PSL was analysed at 1d, 30d and 180d in parallel with three muscles representative of well-known specific patterns of maturation: Masseter (M), Semimembranosus (SM) and Rectus Lateralis (RL).

SDS-PAGE (Fig. 1) and WB (Fig. 2) showed that adult fast 2A and 2X MyHC isoforms, a third fast isoform (likely corresponding to extraocular MyHC) and slow MyHC-1 isoforms were expressed in the extraocular muscles (RL) at all stages of maturation. In addition at 1 day, a developmental isoform, positive to anti-neonatal antibody 2E9 was present. Importantly, antibody 2E9, directed against neonatal myosin showed a cross-reactivity with the slow myosin (Fig. 2). In contrast in the PSL, the prevalent postnatal isoform was the slow (MyHC-1), accompanied at 1 day by a minor amount of a fast isoform (2A) and a barely detectable developmental isoform. At 30d after birth, the slow isoform was the only one detectable and it was clearly reduced at 6 months (180d). As expected, M and SM during development showed a progressive shift, respectively, into slow and fast phenotype.

The pattern of MyHC expression was also confirmed by Real Time PCR analysis (Figs. 3 and 4). The gene expression in SM was taken as the calibrator sample; gene expression analysis showed the maturational transition of masseter (M) towards slow MyHC with only a minor component of the fast isoform 2A still present at 6 months (180d). In the RL the expression of fast isoforms, included the 2B isoform, was persistent at the same stage (180d). In addition, developmental (Embryonic and Neonatal) and extraocular MyHC were always detectable at mRNA level, in partial contrast with their disappearance or low levels as proteins.

In the PSL the slow MyHC mRNA expression was more abundantly expressed at 1d in a significant way, more than in any other muscle, accompanied by a weak signal of the fast 2A isoform and Neonatal

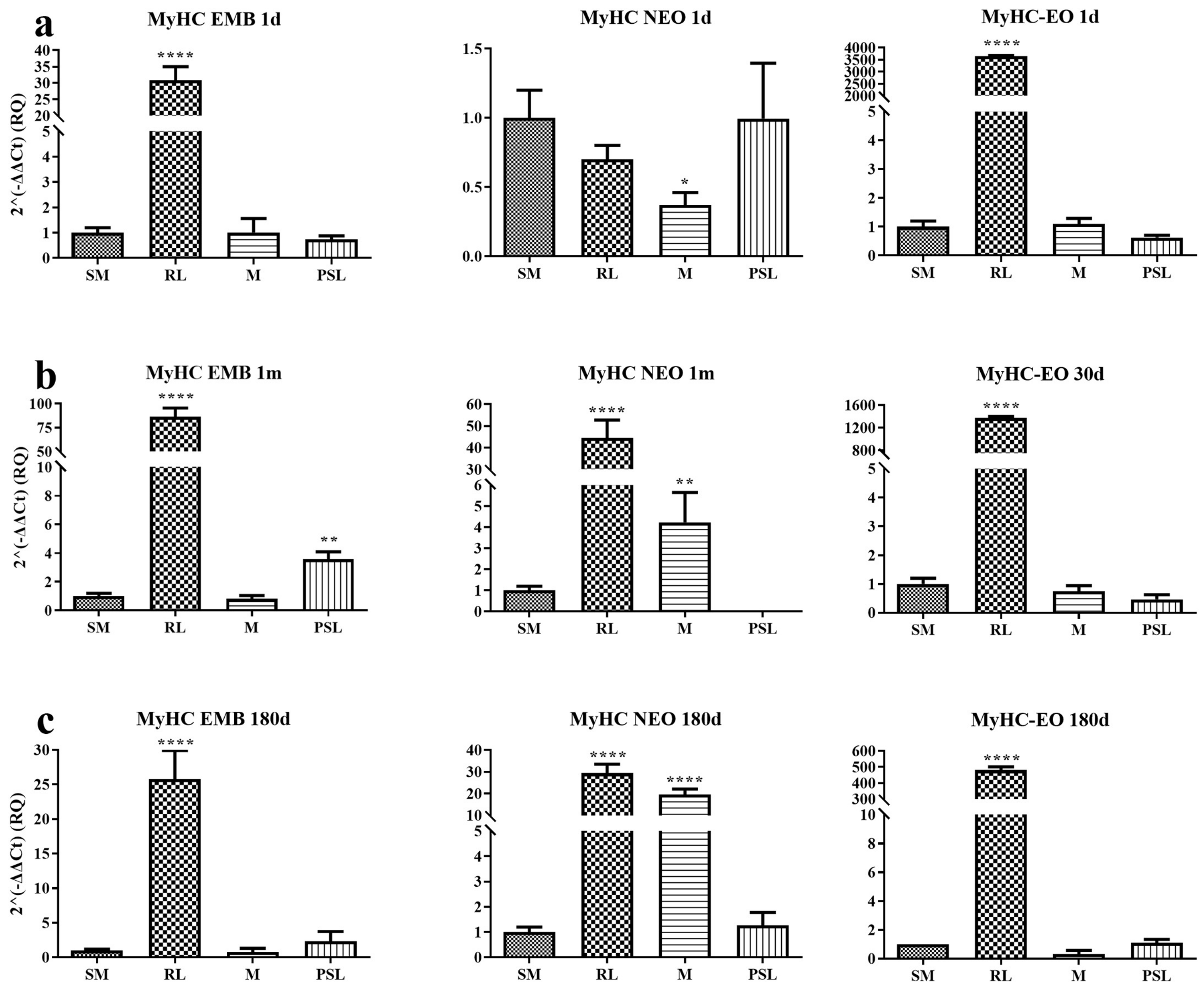


Fig. 4. MyHC isoforms gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of embryonic MyHC (MyHC EMB), neonatal MyHC (MyHC NEO) and extraocular MyHC (MyHC-EO) in the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM, detected by Real Time PCR. SM was used as calibrator sample. $^{*}p < .05$; $^{**}p < .01$; $^{****}p < .0001$.

MyHC. The expression level decreased and at 180d virtually no MyHC transcripts were observed.

3.2. *Expression of myogenic regulatory factors and cellular signalling*

In order to assess the presence of quiescent satellite cells, we determined the expression of Pax7, a molecular marker of satellite cells. We found that Pax7 was expressed in all analysed muscles, at all the time-points (Fig. 5). RL showed the highest expression, if compared to other examined muscles. In the PSL, Pax7 expression was much lower compared to the other muscles with a further significant decrease detected between 30 and 180 d age (Fig. 5).

Next, we determined the expression levels of three important myogenic regulatory factors: MyoD and Myf5, both involved in the muscle determination, and Myogenin, a transcription factor involved in muscle differentiation (Fig. 6). At 1d and 30d, the PSL showed a significant lower expression of MyoD and Myf5 compared to the other muscles (Fig. 6a, b); in contrast, the levels of Myogenin, which controls differentiation, appeared significantly up-regulated in the PSL at 1d and 30d (Fig. 6a, b), thus suggesting an active differentiation of PSL muscle fibres. At 6 months (180d), all myogenic regulatory factors were down-regulated in the PSL in a significant way (Fig. 6c).

The expression of Wnt signalling factors was also explored by Real Time PCR (Fig. 7). The expression of two members of the Wnt family were determined: Wnt1 which is considered to induce satellite cell proliferation and Wnt4 which is considered an inhibitory factor (Otto et al., 2008). Wnt1 showed a significant higher expression in M and SM at 30d only compared to PSL (Fig. 7b), although the expression was significantly higher in RL muscle at all the examined stages (Fig. 7). The PSL showed a significant decrease at 30d (Fig. 7b) and a higher expression at 180d respect than M and SM (Fig. 7c). The relative expression of Wnt4 in PSL showed a progressive decline from 1d to 180d; in particular, at 1d the Wnt4 expression levels were similar among all muscles (Fig. 7a), but at 30d it decreased significantly in the PSL only (Fig.7b). At 180d the RL muscle showed the highest expression while the PSL the lowest one (Fig. 7c).

Insulin-like growth factor 1 (IGF-1) and Myostatin (MSTN) are two signalling molecules involved in the regulation of muscle fibre size: the

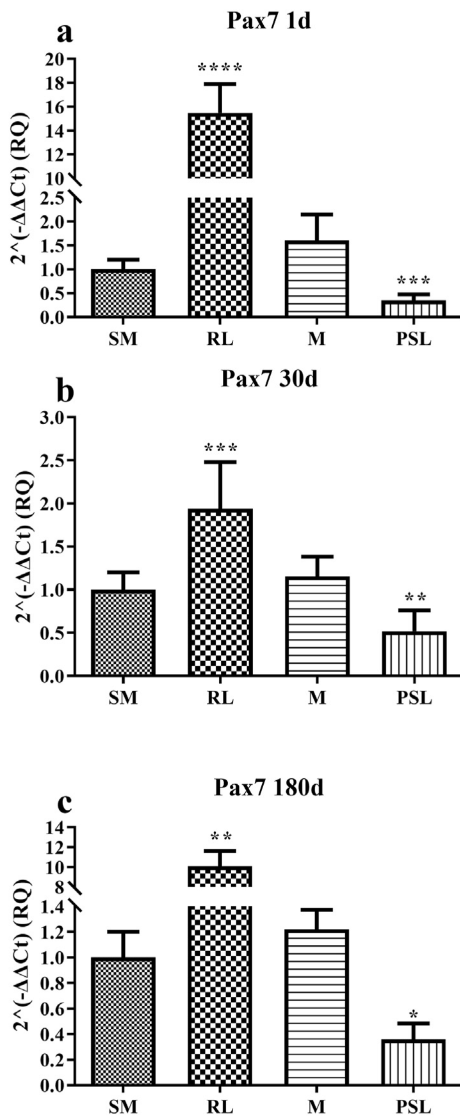


Fig. 5. Pax7 gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of Pax7 in the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM detected by Real Time PCR. SM was used as calibrator sample. $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$.

former with a pro-hypertrophy action and the latter with a pro-atrophy action (Lee and McPherron, 1999; Rommel et al., 2001; Egerman and Glass, 2014; Endo, 2015). The IGF-1 gene expression detected in the PSL showed a significantly higher expression compared to M and SM muscles, at all examined stages (Fig. 8); in parallel to this, a significant lower expression of Myostatin was observed for the PSL, with reference to the calibrator muscle (SM), at all stages (Fig. 8). At 180d SM, PSL and RL showed a significant down-regulation of Myostatin expression.

We also analysed the expression of two signalling molecules, which are not directly involved in controlling maturation and growth of muscle fibres: the vascular endothelial growth factor (VEGF), which is related to angiogenesis, and the transforming growth factor beta 1 (TGF-β) which is considered a powerful pro-fibrotic factor. VEGF gene expression showed that PSL expressed very low levels of this angiogenic factor, especially at 30d and 180d in comparison to the other muscles. The differences were significant compared to the SM (Fig. 8). In contrast, TGF-β1 mRNA level was significantly much higher in the PSL than in all other muscles, in particular at 30d and 180d (Fig. 8b, c).

4. Discussion

The fetlock joint of ungulates is able to work under amazing pressure thanks to some very specific anatomical features, as the PSL, a muscle that changes its structure during its development and becomes a strong fibrous ligament. Indeed, in full contrast with its original postnatal organization, the adult PSL is composed almost exclusively by a fibrous structure that contributes to the storage and release of elastic strain energy during the step cycle and has a suspensory function for the fetlock joint (Jansen et al., 1993). Muscle fibres are virtually absent in the adult animal, but are present and abundant at birth. Our previous study (Mascarello and Rowlerson, 1995) allowed us to reach the following conclusions: 1) an arrested development does not occur and is not the cause of muscle-connective tissue transformation in PSL; 2) the maturation sequence (as determined by fibre type transition) is greatly accelerated, even before birth, in PSL compared to other skeletal muscles; 3) in PSL, muscle fibre degeneration is not followed by muscle fibre regeneration. A very recent investigation on the ultrastructure of PSL (Melotti et al., 2019) showed that the density of satellite cells progressively declines by approximately 10 folds from birth to 6 months of age. Together, these findings point to a specific myogenic program and to an impaired regenerative response in the PSL muscle. The regenerative potential of a muscle mainly depends on the resident satellite cells and their interaction with their local and systemic environment, which together form the so-called “niche” (Yablonka-Reuveni et al., 2015). Conboy and Rando (2005) and Brack et al. (2007), showed that the loss of ability to regenerate muscle has been associated with an increase of fibrogenic activity, a depletion of satellite cells and changes in the Wnt signalling. Pax7 gene expression analysis, performed during the first stages of animal growth (from day 1 to day 180), revealed a progressive decrease of the expression of this factor, which is a marker for the quiescent satellite cells presence in PSL: transmission electron microscopic (TEM) observations have confirmed this trend (Melotti et al., 2019). A decrease density of satellite cells occur in all skeletal muscles during development and is linked to their role as donors of myonuclei (White et al., 2010). As indicated by analysis of Pax7 expression, the decrease in PSL is much greater than in M and SM, but not in the RL. Although satellite cells might not be directly involved at all in the involution of PSL, their number (Melotti et al., 2019) and the Pax7 mRNA decreased drastically in the first six months of life. Furthermore, peculiar fluctuations of the expression of genes involved in the Wnt signalling have been observed. Different research groups have demonstrated that alterations of the Wnt signalling pathway may lead to a modification of the fibre type composition (Strochlic et al., 2012; von Maltzahn et al., 2012; Cheng et al., 2018) but also to a decrease of myogenesis and muscle reparation (Cisternas et al., 2014a). As a matter of fact, Wnt signalling has a key role in skeletal muscle dynamics, especially during myogenesis (Cisternas et al., 2014b). Although Wnts have an important role in these biological processes, Brack et al. (2007) have shown that the satellite cells in the aged niche, influenced by the systemic environment, tend to differentiate to the fibrogenic *lineage* instead of the myogenic *lineage*. This “*lineage* conversion” is supposedly linked to an increase of the canonical Wnt1 signalling (Wnt/β-catenin) in aged mice (Rudnicki and Williams, 2015) and this result resembles what we observed in the PSL of six months (180d) old lambs (i.e. low expression of Pax7 and a high expression of Wnt1). This hypothesis is also supported by the low, almost absent, mRNA levels of Wnt4 that we have detected at 30 and 180 days in the PSL. In fact, this molecule is known to be down-regulated during processes of fibrosis in the muscle tissue (Cisternas et al., 2014a) as it is usually expressed in resting muscles (von Maltzahn et al., 2012).

The three myogenic regulatory factors analysed in the present study are crucial for muscle tissue differentiation and determination during embryonic and fetal development, and for its regeneration processes after birth (Endo, 2015; Asfour et al., 2018). While Myf5 and MyoD showed a low level of gene expression from day 1 to day 180, Myogenin

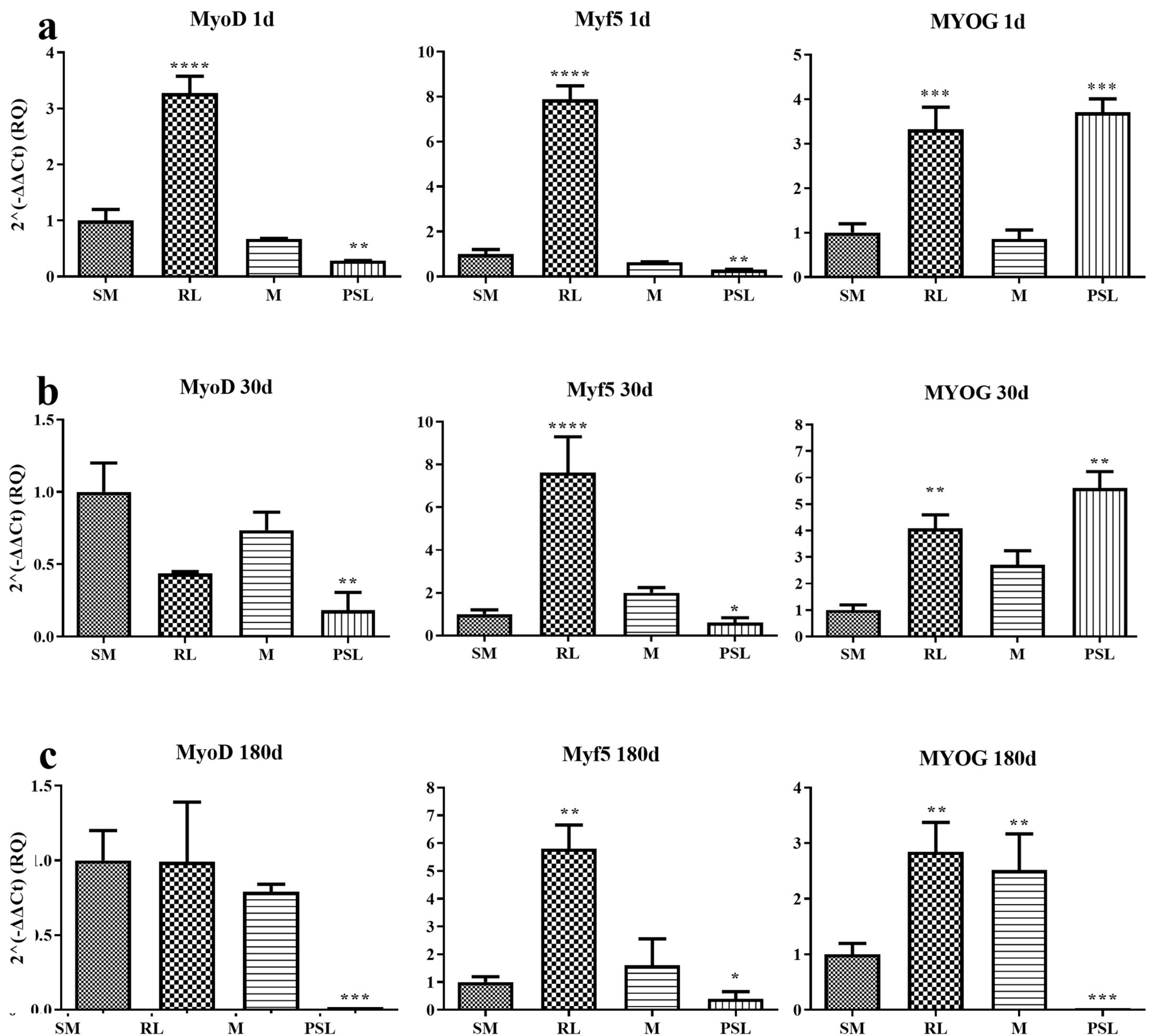


Fig. 6. Myogenic regulatory factors gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of MyoD, Myf5 and Myogenin (MYOG) in the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM detected by Real Time PCR. SM was used as the calibrator sample. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$.

was upregulated at 1 day and 30 days after birth. The expression of Myogenin is fundamental for the differentiation of myoblasts also during muscle regeneration (Endo, 2015) and concomitantly its expression inhibits Pax7 expression (Halevy et al., 2004; Zammit et al., 2004), like we observed at 1 month (30d). This result is also supported by the fact that IGF-1, which impinges on muscle differentiation (Mourkioti and Rosenthal, 2005; Ten Broek et al., 2010; Jiménez-Amilburu et al., 2013), was highly expressed at 30 days in PSL compared to other muscles (M and SM). It seems that the PSL is precociously involved in a muscle remodeling phase with a high expression of Myogenin and IGF-1 together with a low expression of Pax7 and MyoD, absent in other muscles (M and SM). The latter expression pattern profile might be considered as an activating bell of the transitional phase towards the fibrosis fate.

The data on MyHC isoforms expression confirmed the view that the transition towards a slow phenotype occurred much earlier in PSL than in other muscles. Actually, in this study, PSL was compared with three ovine muscles with a very specific adult fibre type pattern: Masseter which as all ruminant becomes a predominantly slow muscle, Semimembranosus which in adult has a rich fast fibres population and Rectus lateralis which, like all extraocular muscles, is characterized in its adult stage by the presence of a wealth of different fibre types (Mascarello et al., 2016). The relevant changes in MyHC isoforms, which are the markers of fibre types, are easily detectable in the data obtained in this study comparing 1, 30 and 180 days. Evidence from the present data suggests that, in PSL, fast muscle fibres probably undergo a sort of degenerative process much earlier than in any other muscle here considered. This evolution towards a completely slow phenotype is not peculiar to PSL only. In fact, different studies in a variety of species, demonstrated that a decreased expression of fast and an increased

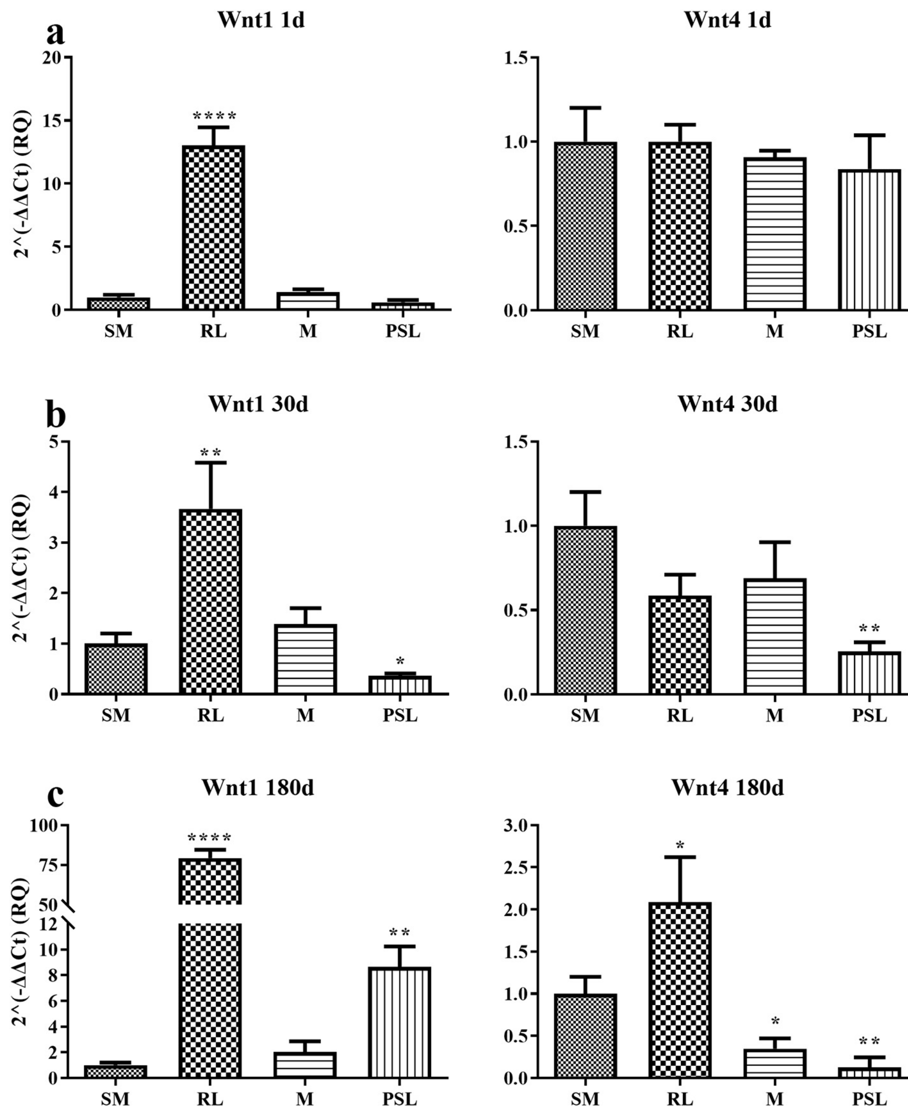


Fig. 7. Wnts gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of Wnt1 and Wnt4 in the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM detected by Real Time PCR. SM was used as calibrator sample. *$p < .05$; **$p < .01$; ****$p < .0001$.

expression of slow MyHC occurred in many physiological situations including ageing (Klitgaard et al., 1990; Barberi et al., 2015; Muroya et al., 2013; Talbot and Maves, 2016). This is attributed to a preferential atrophy of fast fibres and the enlargement of slow motor units by re-innervation of denervated fast fibres. Moreover, concomitantly, satellite cells progressively disappear, instead of being activated in order to start a regenerative process.

The high levels of IGF-1 found in the PSL, together with the low values of Pax7, should remark the intense and precocious cellular activity that occurs in this muscle and, therefore, the massive myogenesis that is ongoing during the first postnatal weeks with a still active differentiation process dictated by Myogenin (Endo, 2015). In the meantime, the generation of new muscle cells is declining and this is may be due to a low numbers of satellite cells. In this context, the IGF-1 intense expression observed in PSL at 180 days might play a role in the proliferation and stimulation of fibroblasts (Luo et al., 2013) instead of muscle cells. Alongside, also factors of the TGF-β superfamily are known to act as pro-fibrogenic molecules (Xu et al., 2018). TGF-β1 has the capacity to attract fibroblasts and to stimulate their proliferation (influencing intramuscular fibroblasts already present in the tissue as well). This molecule has the same effects also towards mesenchymal stem cells, inducing their differentiation into fibroblasts. All these effects result into stimulation and deposition of extracellular matrix proteins (Li et al., 2004; Cencetti et al., 2010; Wan et al., 2012). Hence, it is not surprising that high levels of TGF-β1 were detected in the PSL during its transformation towards a connective fate. Both molecules (TGF-β1 and IGF-1) stimulate the deposition of collagen, and their upregulation may be due to mechano-transduction mechanisms. As a matter of fact, the PSL undergoes a mechanical stress since the first day after birth (Wilson et al., 1995; Kjaer, 2004).

In conclusion, the present results provide the first description of the signalling network, which supports the rapid involution of the muscle component of the ovine PSL. The transition of the expression of the myogenic factors induced an accelerated maturation, which is confirmed by the transition of MyHC isoform expression. The loss of regenerative potential is related to the low Pax7 gene expression and the altered Wnt signalling. High TGF-β1 expression is likely to be responsible of the activation of fibrosis. It remains to establish whether all these changes are induced by a muscle-specific transcriptional program or are triggered by the peculiar loading conditions on PSL. The transcriptional diversity documented at day 1, i.e. just at the first day of loading of PSL, is suggestive that an intrinsic muscle specific regulation

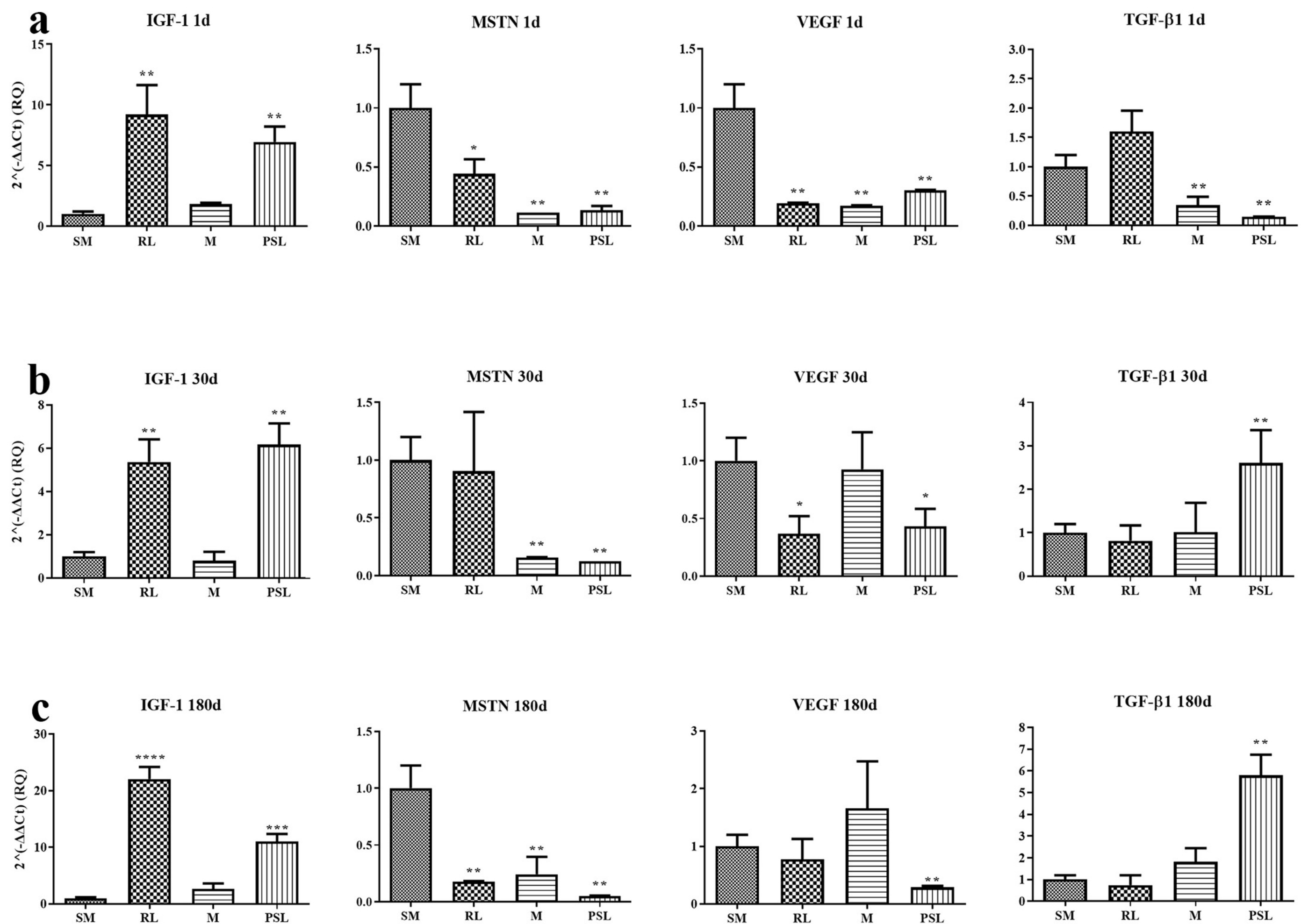


Fig. 8. IGF-1, TGF-β1, VEGF and MSTN gene expression analysis at different ages. Relative quantification (RQ) of the gene expression of insulin-like growth factor (IGF-1), transforming growth factor beta 1 (TGF-β1), myostatin (MSTN) and vascular endothelial growth factor (VEGF) in the rectus lateralis extraocular muscle (RL), masseter muscle (M) and proximal sesamoidean ligament (PSL) respect than the semimembranosus muscle (SM) at (a) 1, (b) 30 and (c) 180 days after birth (1d, 30d and 180d respectively). Data are shown as the mean + SEM detected by Real Time PCR. SM was used as calibrator sample. *p < .05; **p < .01; ***p < .001; ****p < .0001.

is responsible. Overall, the PSL should not be considered as a muscle that is prematurely ageing but, instead, as a muscle that is undergoing a very specific differentiation process which surely implies myofiber involution.

Supplementary data to this article can be found online at …

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