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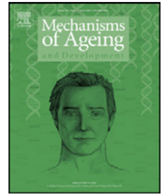

Transcriptional profiling of skeletal muscle reveals factors that are necessary to maintain satellite cell integrity during ageing

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ABSTRACT

Skeletal muscle ageing is characterized by faulty degenerative/regenerative processes that promote the decline of its mass, strength, and endurance. In this study, we used a transcriptional profiling method to better understand the molecular pathways and factors that contribute to these processes. To more appropriately contrast the differences in regenerative capacity of old muscle, we compared it with young muscle, where robust growth and efficient myogenic differentiation is ongoing. Notably, in old mice, we found a severe deficit in satellite cells activation. We performed expression analyses on RNA from the gastrocnemius muscle of young (3-week-old) and old (24-month-old) mice. The differential expression highlighted genes that are involved in the efficient functioning of satellite cells. Indeed, the greatest number of up-regulated genes in young mice encoded components of the extracellular matrix required for the maintenance of the satellite cell niche. Moreover, other genes included Wnt inhibitors (*Wif1* and *Sfrp2*) and Notch activator (*Dner*), which are putatively involved in the interconnected signalling networks that control satellite cell function. The widespread expression differences for inhibitors of TGFbeta signalling further emphasize the shortcomings in satellite cell performance. Therefore, we draw attention to the breakdown of features required to maintain satellite cell integrity during the ageing process.

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1. Introduction

Ageing is a physiological process that includes a gradual decrease in skeletal muscle mass, strength, and endurance coupled with an ineffective response to tissue damage (Degens, 2007). Muscle fibre loss is counteracted by satellite cells; the predominant adult myogenic progenitors located underneath the basal lamina of myofibres, that are capable of lifelong maintenance and repair of skeletal muscle (Hawke and Garry, 2001; Shi and Garry, 2006). Studies on primary satellite cells have shown that oxidative stress results in a loss of viability, a shorter lifespan, and a marked decrease in their proliferative capacity (Renault et al., 2000). Indeed, adaptations of antioxidant defence systems are crucial to deal with the increased generation of reactive oxygen species (ROS) during muscle regeneration in exercise and injury (Ji, 2008; Pierce et al., 2007).

The functioning of satellite cells depends on external cues imparted by the surrounding environment (niche) and their inherent ability to undergo myogenesis (Collins et al., 2005). In ageing, it is not clear whether the deficient performance of the cells is a consequence of an altered niche, or an intrinsic cellular insufficiency, or both. Experiments have shown that the old muscle niche fails to support effective muscle regeneration. However, when old injured muscle is placed in a young host by cross-muscle grafting or when it is exposed to a young systemic niche by parabiotic pairings of old and young animals, regeneration is highly efficient (Brack et al., 2007; Carlson and Faulkner, 1989; Conboy et al., 2005). On the other hand, other studies have pointed to an intrinsic shortcoming of satellite cells to undergo myogenesis during ageing (Beccafico et al., 2007; Lees et al., 2006).

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The niche is made up of the myofibre including its basal lamina, components of the extracellular matrix (ECM), and supporting cells from the microvasculature and interstitia (Gopinath and Rando, 2008). The ECM contributes to muscle regeneration. Transcriptional profiling has shown that ECM components are significantly up-regulated in regenerating skeletal muscle and during myoblast differentiation (Goetsch et al., 2003; Moran et al., 2002) and significantly down-regulated during ageing (Pattison et al., 2003b). One role for the ECM is to make available extracellular molecules emanating from the host fibres, the supporting cells from the microvasculature and/or satellite cells. This is essential for mediating signal transduction networks, which are altered in age-related pathologies. Due to the complexity of crosstalk and integration, it is not completely understood how these networks alter their signals during the ageing process. Some of the affected pathways are influenced by Notch signalling, whose age-related decrease has been shown to reduce the regenerative potential of muscles (Conboy et al., 2003). For example, transforming growth factor beta (TGFbeta) signalling, which increases and interferes with the regenerative capacity in old muscle, is attenuated by Notch activation (Carlson et al., 2008a). Moreover, increased Wnt activity, which alters the fate of stem cells and increases fibrosis during ageing, is antagonized by Notch activation (Brack et al., 2008, 2007).

To obtain a better understanding of the molecular basis of age-related problems, gene expression profiling technology has made it possible to assess the expression levels of thousands of genes. In this way, discrepancies in the events involved in regulating biological processes at the transcriptional level can be illustrated. Several studies have screened for differences in gene expression in adult and old skeletal muscle of rodents and humans (Edwards et al., 2007; Giresi et al., 2005; Jozsi et al., 2000; Kayo et al., 2001; Lee et al., 1999; Pattison et al., 2003a,b; Welle et al., 2000, 2001, 2003). In addition, other genome-wide screens related to deficiencies in ageing such as muscle atrophy (Batt et al., 2006; Chen et al., 2007; Dapp et al., 2004; Jagoe et al., 2002; Kostrominova et al., 2005; Lecker et al., 2004; Nikawa et al., 2004; Paoni et al., 2002; Raffaello et al., 2006) and regeneration (Fluck et al., 2005; Goetsch et al., 2003; Zhou et al., 2006) have also revealed similar differences. Gene expression analyses of ageing have compared two distinct stages of muscle development, that is, adult muscle, which is typically quiescent with few degenerating/regenerating muscle fibres, and old muscle, which undergoes mainly degeneration with a fairly faulty regenerative capacity. These studies have revealed differences in genes involved in maintaining muscle integrity such as DNA damage repair, stress responses, reinnervation, immune/inflammatory responses, RNA binding and splicing, and proteasome degradation.

However, another stage of postnatal muscle development that is distinct from both adult and old muscles occurs in young mice after weaning (Hawke and Garry, 2001; Shi and Garry, 2006). It is a rapid growth stage characterized by the proliferation and differentiation of satellite cells that contribute progeny that fuse with the enlarging myofibres. The number of satellite cells in rodent hindlimbs, which make up approximately 30% of total muscle nuclei in young mice, decreases to approximately 4% in adult mice and 2% in old mice (Snow, 1977).

It is thus possible that genes are expressed or repressed in young mice in order to promote continuous muscle maturation rather than the degenerative state observed during ageing. Hence, a more appropriate approach to unveil significant gene expression differences with respect to deficits in regeneration components and the causes of degeneration in old animals would be to compare old muscle with young muscle rather than with adult muscle. To our knowledge, no gene expression studies have compared young rapidly growing muscle that is actively developing to old muscle that is undergoing an opposite course of action, i.e. degenerating. Our results show that genes involved in efficient satellite cell function are differentially up-regulated in young muscle. This includes various components of the ECM that make up the satellite cell niche as well as critical signalling factors such as Notch activators, Wnt inhibitors, and TGFbeta attenuators, which likely play a role in the ageing process. Our screen also show that expression of certain metabolic factors involved in reducing the oxidative stress that hinders satellite cell proliferation is differentially regulated.

The present study highlights an impairment of satellite cell function in old muscle by assessment and annotation of gene expression differences with young mice. Our novel experimental approach provides insight into proteins that are potentially involved in cellular processes and signalling networks and that may be essential for the proper maintenance of satellite cells. Understanding how these factors function in different pathways and processes may open up new avenues for the development of therapies to prevent satellite cell function deficits in ageing.

2. Materials and methods

2.1. Animals

Young (3-week-old), adult (12-week-old) and old (24-month-old) male C57-Bl/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). After sacrifice, the gastrocnemius (Gas) muscles were immediately collected, weighed, and processed for histological examinations, or were flash-frozen for molecular analyses. Flash-frozen muscles were mechanically crushed in liquid nitrogen using a mortar and pestle and stored at −80 °C until used to extract RNA. All procedures involving animals followed "Principles of laboratory animal care" (NIH publication no. 86-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee of Université de Sherbrooke (Protocol # 133-06).

2.2. Histology and immunofluorescence

Gas muscles were immersed in successive baths of PBS containing increasing concentrations of sucrose (5%, 15%, and 30%) and marked mid-length with China ink. The tissues were then embedded in 30% sucrose:Tissue-Tek® O.C.T.™ (1:2) (Somagen, Edmonton, AB, Canada), frozen in isopentane chilled in liquid nitrogen, and stored at −80 °C until used. Cryosections (5 μm) were collected starting from the middle of each muscle (ink mark), which is where the girth of the muscle is greatest. The cross-sectional area (CSA) analysis of the myofibres was performed on H&E-stained sections. Over 150 myofibres from at least 10 different fields (40×) were measured using ImageJ software (NIH, Bethesda, MD, USA). CSA of muscle myofibres are represented as a distribution histogram with a non-linear regression curve.

For co-staining immunofluorescence experiments, air-dried cryosections were fixed in 2% PFA (10 min RT), blocked and permeabilized in PBS–5% goat serum–5% BSA containing −0.01% Tween®20. They were then incubated with rabbit anti-MyoD (1:500) (SantaCruz M-318, Santa Cruz, CA, USA) or rabbit Ki67 (1:2) (Thermo Scientific, SP6, Fremont, CA, USA), mouse Pax7 (1:2) (DSHB) and rat laminin 2 (1:600) (Sigma) primary antibodies followed with Alexa Fluor® 594-conjugated goat anti-rabbit IgG (1:2000) and Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:2000) and goat anti-rat IgG Alexa Fluor® 647 (1:2000) secondary antibodies (Invitrogen, Burlington, ON, Canada), respectively. Cell nuclei were labelled with DAPI reagent (Sigma–Aldrich, Oakville, ON, Canada) added for 10 min after secondary antibody incubation. Sections were viewed using a TE-2000-S epifluorescence microscope (Nikon, Mississauga, ON, Canada). All MyoD, Pax7, MyoD/Pax7 and Ki67/Pax7 positive cells were enumerated on the entire transversal section of 3–4 gastronemius muscles at their mid point. For embryonic myosin heavy chain positive fibres gastrocnemius muscle sections were stained with anti-embMyHC (1:10; F1.652, DSHB) without prior fixation.

2.3. Quantitative PCR

Total RNA was extracted from flash-frozen crushed muscles using TRIzol® (Invitrogen). The RNA (1 μg) was reverse-transcribed using Reverse Transcriptase Superscript II (Invitrogen). QPCR was performed using 50 ng of cDNA under the following conditions: a 5 min denaturation step at 95 °C, followed by 40 cycles of 40 s at 95 °C, 40 s at 56 °C, and 40 s at 72 °C. qPCR assays were performed on a Rotor-Gene 6000 (Corbett Robotics, Eight Mile Plains, Australia) using iQSYBR Green Supermix (BioRad, Mississauga, ON, Canada). The results were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method normalized to HPRT1. The primer sets are listed in Supplemental Table 1. All data are expressed as means ± SEM. Unpaired *t*-tests were used to compare groups of mice. $p < 0.05$ was considered to be statistically significant.

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2.4. Microarray and data analyses

Total RNA was extracted from 10 mg of crushed frozen Gas muscles from young and old mice using TRIzol® (Invitrogen). The quality of the RNA was assessed using an Agilent Bioanalyzer (Agilent Technologies Canada Inc., Mississauga, ON, Canada). cRNA generated from the mRNA was hybridized to Affymetrix GeneChip Mouse Expression MOE430 2.0 (Affymetrix, Santa Clara, CA, USA, http://www.affymetrix.com), which contains 45,153 probe sets to analyze the expression levels of over 39,000 transcripts and variants from over 34,000 well-characterized mouse genes. Three samples each of young and old muscles were analyzed as described in the Affymetrix GeneChip Protocol (Affymetrix) using a GeneChip® Scanner 3000 (Affymetrix).

The triplicate expression data sets were processed as follows. The MAS5 expression and present calls were derived from the CEL files using the Bioconductor MAS5 implementation. For the broad analysis of gene function changes, the MAS5 signal data replicates were averaged by sample. MAS5 calls were collated by majority vote, that is, a probe set was considered to have a *p* call (Present) if a *p* call was generated in at least two of the three data sets. Otherwise, it was considered absent.

For other fold change analyses, we normalized the Affymetrix CEL files using the GCRMA (Tusher et al., 2001; Wu et al., 2004) implementation in Bioconductor http://www.bioconductor.org/docs/faq/). We used the Bioconductor siggenes implementation of the SAM algorithm to identify probe sets whose signals in the young and old Gas data sets were significantly different. A delta of 4 was used and the false discovery rate (FDR) was 11.6%.

The gene name, gene symbol, and GO terms corresponding to each probe set were retrieved from the NetAffx (Liu et al., 2003) annotation database (https://www.affymetrix.com). Additional GO terms were obtained by examining related databases using automated data mining procedures. Raw microarray data are available online at the NCBI Gene Expression Omnibus (Barrett et al., 2009) (GEO, http://www.ncbi.nlm.nih.gov/geo/), GEO Series Accession No. GSE14678.

2.5. Cell and muscle fibre isolation

Single myofibres from the gastrocnemius muscles of four young, adult and old mice were isolated by collagenase digestion and pooled as previously described (Rosenblatt et al., 1995). Primary myoblasts were isolated from hind limb muscles and cultured on collagen-coated Petri dishes in HamF10 supplemented with 20% fetal bovine serum (FBS; Hyclone) (Megeney et al., 1996). For differentiation of myoblasts into myotubes, medium was replaced by Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum for 3 days.

Stromal cells were isolated and analyzed immediately or cultured as previously described (Grenier et al., 2007; Scime et al., 2005). Briefly, gastrocnemius muscle was minced and digested in collagenase I (Sigma) for 45 min. The tissue slurry was washed with DMEM containing 10% FBS and poured through a 100-μm and then a 50-μm cell strainers prior to plating or immediate analysis (BD Falcon, Mississauga, Canada).

2.6. Western blot analysis

Muscles were crushed and homogenized in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5) containing a protease inhibitor cocktail Complete™ (Roche Molecular Biochemical, Laval, QC, Canada). The homogenate was clarified at 18.8 g for 10 min at 4 °C and the supernatant was recovered. Protein concentrations in the supernatant were determined using Bradford's method (BioRad). Protein extracts (50 μg) were separated on a 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were then incubated overnight at 4 °C in PBS-T (3.5 mM NaH_2PO_4, 17.4 mM Na_2HPO_4, 3.5 mM KCl, 137 mM NaCl containing 0.1% Tween-20) with primary antibodies. The antibodies used are listed in Supplemental Table S2. After washing, immune complexes were incubated for 1 h with anti-rabbit (1:4000; BioRad, Mississauga, ON, Canada) or anti-goat (1:20,000; Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) HRP-conjugated secondary antibodies. The membranes were scanned and the bands were quantified by densitometric determination using ImageJ software (NIH, Bethesda, MD, USA).

2.7. Muscle injury

Muscle injury was induced by injecting 40 μl of 10 μM cardiotoxin (CTX; Latoxan, Valence, France) into both side of one gastrocnemius muscle per mouse as previously described (Caron et al., 2009; Grenier et al., 2007). As control, saline was injected into the contralateral gastrocnemius muscles. At 3, 10 and 14 days post-injury, gastrocnemius were harvested.

2.8. Statistical analyses

Muscle wet weights were expressed as means ± SEM, significance was established by unpaired two-tailed Student's *t*-test with a confidence interval of 95% (*p* value <0.05). For CSA of muscle myofibres statistics of median and interquartile range were used. Statistical significance was assessed using Anova analysis followed with a Tukey post-test with a confidence interval of 95% (*p* value <0.05). For the cardiotoxin injury experiments significance was established using paired two-tailed Student's *t*-test. GraphPad Prism 5.0 software™ was used for all statistical analyses.

3. Results

3.1. Experimental animals

We compared the gene expression profiles of muscle from young (3-week-old) and old (24-month-old) mice. We confirmed that young and old mice represent two distinct stages of postnatal muscular development. This was accomplished by highlighting differences in their musculature with that of adult (3-month-old) mice representing the commonly studied postnatal stage of development characterized by its maturity and absence of growth. As expected, we observed a significant increase in the mass of the gastrocnemius (Gas) muscles of adult and old mice compared to the Gas of young mice ($p < 0.0001$), suggesting that the young muscle was undergoing growth and had not reached maturity (Fig. 1a). In addition, we found a significant decrease in old compared to adult Gas muscle mass ($p < 0.002$), indicating that it was experiencing an age-related deficit (Fig. 1a). These results were corroborated by the distribution of the cross-sectional areas (CSA) of the Gas muscle fibres, which revealed that the fibres of young muscle were smaller than those of adult and old muscles (Fig. 1b). Statistical analysis determined that young tissue CSA median is 667.5 μm^2 with an inter quartile range (IQR) of 445 μm^2, adult tissue CSA median is 1860 μm^2 with an IQR of 1209 μm^2 and old tissue median is 1267 μm^2 with an IQR of 898.8 μm^2 (Fig. 1b). In addition, the fibre CSAs of old muscle had a more heterogeneous fibre size distribution than that of adult muscle.

We also assessed the activity of myogenic progenitor cells in our experimental groups by determining satellite cell activity in Gas

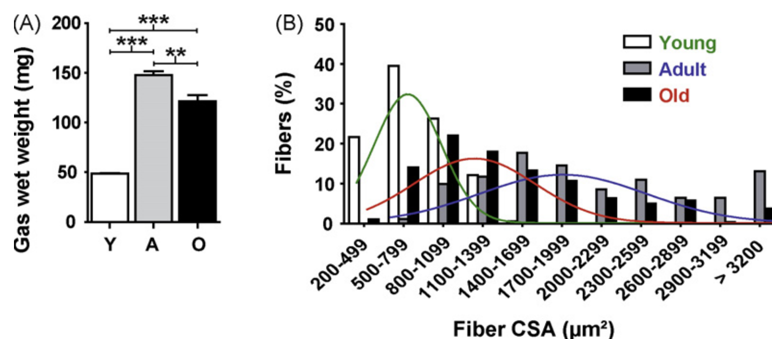

Fig. 1. Young, adult, and old muscles represent distinctive stages in skeletal muscle development. (a) Graphical representation of wet weights of young (Y) ($n = 8$), adult (A) ($n = 7$), and old (O) ($n = 3$) gastrocnemius (Gas) muscles. (b) Graphical representation of the cross-sectional area (CSA) distribution of young, adult, and old Gas fibres and frequency distribution with non-linear regression curve for the myofibre CSA of young (green), adult (blue) and old (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

muscles during the three stages of growth by co-staining for MyoD and Pax7 (Fig. 2a). The various myogenic cell fractions represented by quiescent Pax7^{+}MyoD^{-}satellite cells, Pax7^{+}MyoD^{+}activated satellite cells (proliferating myogenic precursor or MPC) and Pax7^{-}MyoD^{+}fusion competent MPCs were enumerated (Grenier et al., 2007; Olguin et al., 2007; Zammit et al., 2004, 2006). Our results revealed that young muscle had significantly more Pax7^{+}MyoD^{+} cells representing activated satellite cells than both adult and old muscle (Fig. 2a). There were significantly higher ($p < 0.0005$) activated myogenic progenitor cells in young (1.13%) than in adult (0.24%), moreover significantly higher (10-fold) in adult (0.24%) as compared to old (0.025%) muscles ($p < 0.0014$). These results suggest that there is a severe activation deficiency of satellite cells in old mice. We also assessed the fraction of activated MPCs that were proliferating by analyzing the percentage of Pax7 cells that were positive for the proliferation marker, Ki67 (Fig. 2b).

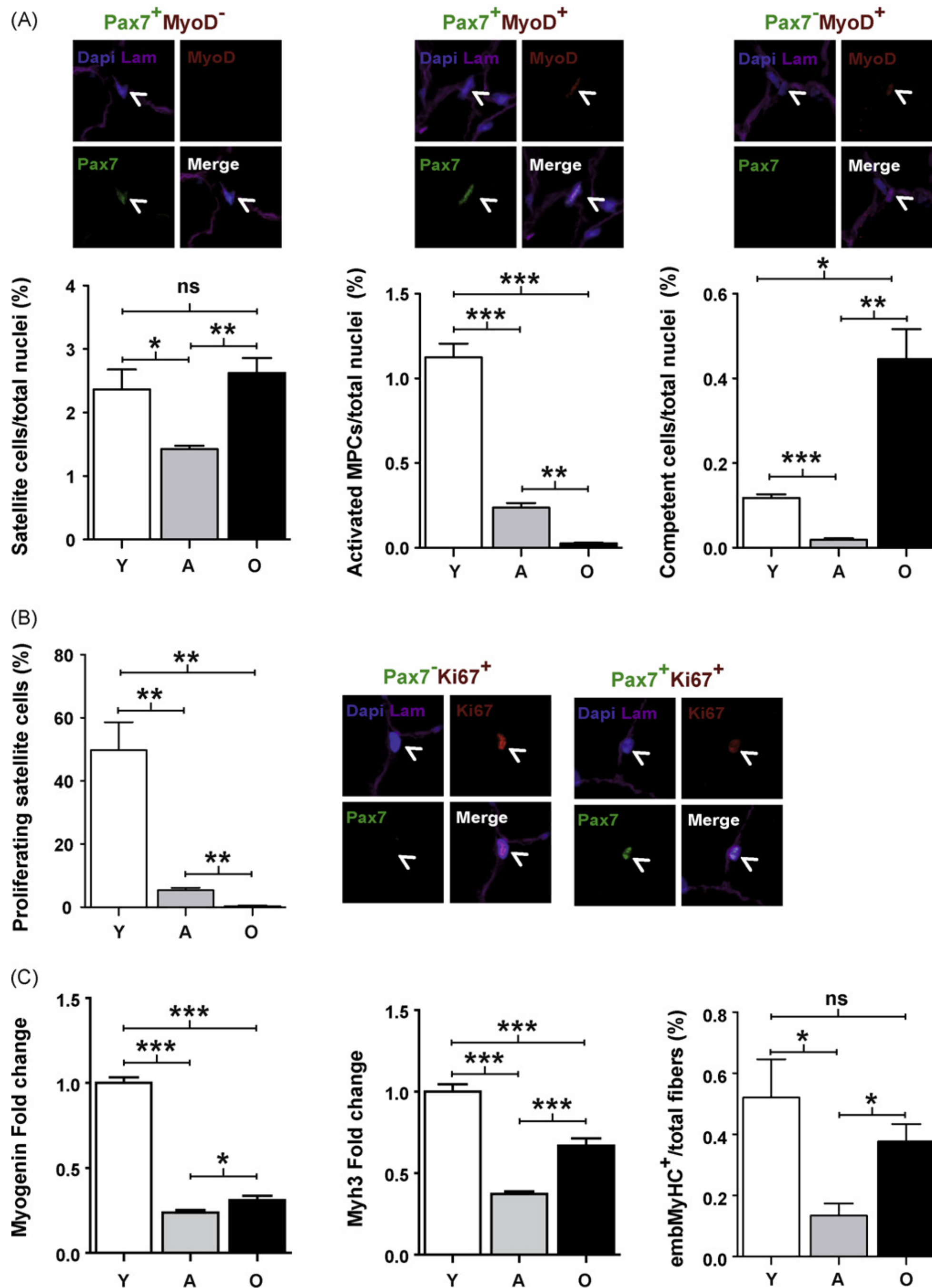

Fig. 2. Young, adult, and old muscles have skeletal muscle characteristics. (a) Representative immunofluorescence for MyoD, Pax7, laminin (Lam) and DAPI nuclear staining of gastrocnemius muscle section, and graphical representation of the number of Pax7^{+}MyoD^{-}, Pax7^{+}MyoD^{+} and Pax7^{-}MyoD^{+} cells per total nuclei. Chevrons denote Pax7^{+}MyoD^{-}, Pax7^{+}MyoD^{+} and Pax7^{-}MyoD^{+} cells ($n = 3$; $^{*}p = 0.05–0.001$; $^{**}p = 0.0009–0.0001$; $^{***}p < 0.0001$). (b) Representative immunofluorescence for Ki67, Pax7, laminin (Lam) and DAPI nuclear staining of gastrocnemius muscle section and graphical representation of the number of quiescent Pax7^{+}Ki67^{-} and proliferating Pax7^{+}Ki67^{+} cells. Chevrons denote Pax7^{+}Ki67^{-}and Pax7^{+}Ki67^{+} cells ($n = 3$; $^{**}p = 0.0009–0.0001$). (c) Quantitative PCR (qPCR) analysis of myogenin and embryonic myosin heavy chain 3 (Myh3), and graphical representation of the number of positive embryonic myosin heavy chain (embMyHC) stained fibres for young (Y), adult (A) and old (O) gastrocnemius muscle. For qPCR young ($n = 4$), adult ($n = 5$), and old ($n = 5$), and for embMyHC stained fibres young ($n = 3$), adult ($n = 4$), and old ($n = 4$) ($^{*}p = 0.05–0.001$; $^{**}p = 0.0009–0.0001$; $^{***}p < 0.0001$).

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We found that young mice had approximately 49.4% of their Pax7 positive cells that were proliferating. Whereas the activation of MPCs leading to proliferation was severely curtailed with aging and dramatically impaired in old mice with only 0.34% of Pax7 cells that were Ki67 positive. This is emphasized by old muscle that had significantly more $Pax7^{-}MyoD^{+}$ fusion competent cells than both young and adult muscle suggesting that old MPCs preferentially become fusion competent rather than proliferate. These results suggest that immature young muscle is actively growing made up of many activated proliferative MPCs as opposed to old muscle that have significantly reduced numbers of activated MPCs that are characterized by a proliferative insufficiency.

Our results also indicate that there is no satellite cell number deficiency in old tissues. The percentage of quiescent satellite cells ($Pax7^{+}MyoD^{-}$) on total nuclei was higher in young and old muscles as compared to adult tissue and was not significantly different between young and old (Fig. 2a).

Taken together, these results revealed that satellite cell number remains constant and regeneration is ongoing in the old muscle compared to the adult, but satellite cell activation and proliferation were severely compromised compared to the young and adult muscle affecting the regenerative capacity.

The dynamic nature of the young versus old muscles, growing and regenerating/degenerating respectively, was confirmed by quantitative PCR (qPCR). Significant high expression levels of myogenin (Myog) and embryonic myosin heavy chain 3 (Myh3), which are markers of differentiating myoblasts and maturing myofibres, respectively, were apparent in Gas (Fig. 2b) (Nakagawa et al., 2005). We also enumerated the fibres that were positively stained for embryonic myosin heavy chain (embMyHC) in gastrocnemius muscle tissue sections. We found significantly more maturing positive embMyHC fibres in the old muscle compared to the adult but significantly less than in the young (Fig. 2c). Although the regenerative character of the young and old muscles was similar, it underscores muscle maturation in the young versus regeneration in old mice. Taken together, these results show that our experimental groups represented two distinct stages of postnatal development, i.e., immature growing (young) and degenerating and inefficiently regenerating (old) muscle that is separate from the mature and stable (adult) muscle used in all previous microarray studies.

3.2. Microarray analysis

To gain new insights in age-related muscle deficits, we performed gene expression analyses of Gas muscles from old ($n = 3$) and young ($n = 3$) mice using Mouse MOE430 v2.0 high resolution arrays (Affymetrix) that contain 45,147 probe sets to analyse the expression levels of over 39,000 transcripts. MAS 5.0 analysis ($p < 0.04$) of mRNA expression revealed that 4531 probe sets had a call "present" (detected gene expression) in both young and old muscles, while 1675 were present only in young muscle and 459 only in the old muscle.

To identify the most significant differentially expressed genes, we applied a Statistical Analysis of Microarray (SAM) analysis (Tusher et al., 2001) using a delta of 4, which gave a false discovery rate (FDR) of 11.6% (Fig. 3). We found 114 probe sets, which corresponded to 99 genes that were significantly differentially expressed in young and old muscles (Fig. 3 and Suppl. Table S3). Surprisingly, 98 of the genes were expressed at a lower level in old muscle, and only one was expressed at a higher level (Suppl. Table S3). For the 99 genes, the fold change expression measured by microarray ranged from 168.5 for *Procollagen, type I, alpha I* (*Col1a1*) to 1.3-fold for *Lipoma HMGIC fusion partner like 2* (*Lhfp12*). Most importantly, 88 of the genes had a differential expression exceeding 2-fold, with 48 exceeding 5-fold.

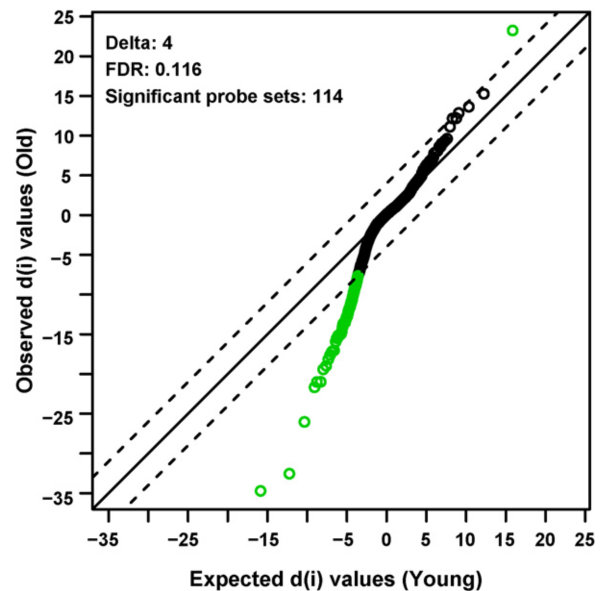

Fig. 3. Identification of significantly differentially expressed genes in young and old muscles. Significance Analysis of Microarray (SAM) scatter plot of the observed relative difference $d(i)$ of old Gas versus the expected relative difference $d(i)$ of young Gas. The solid diagonal line indicates the line for Observed $d(i)$ = Expected $d(i)$, where the relative difference between old Gas and young Gas is identical. The diagonal dotted lines are set at a distance of Delta = 4 from the solid line. One hundred and fourteen probe sets were called significant with a False Discovery Rate (FDR) of 11.6%. The green circles represent the significant probe sets. The black circles represent non-significant probe sets. Raw microarray data are available online at the NCBI Gene Expression Omnibus (Barrett et al., 2009) (GEO, http://www.ncbi.nlm.nih.gov/geo/), GEO Series Accession No. GSE14678. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

We classified the 99 significantly expressed genes in 12 biological categories based on function by Gene Ontology (Ashburner et al., 2000) (Fig. 4). The majority (60%) of the genes were in categories with functions related to muscle growth and development, which is ongoing in young mice. The "extracellular matrix and cell adhesion" category contained the greatest number of genes, i.e., 22, including *Co11a1*, the gene with the greatest fold change. The only gene from the 99 that was significantly down-regulated in young muscle, *proviral integration site 1* (*Pim1*), was in the "cell cycle" category.

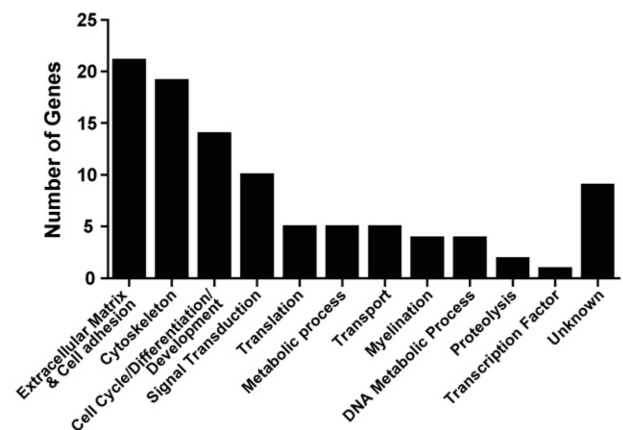

Fig. 4. Classification of the 99 significantly differentially expressed genes in young and old gastrocnemius muscles. The biological classification into 12 categories was based on a function using the GO-slim cut down version of the Gene Ontology (Ashburner et al., 2000).

Thirty percent of the significant differentially expressed genes in the young and old muscles have also been reported to be differentially expressed in adult and old muscles in other microarray studies (Edwards et al., 2007; Lee et al., 1999; Pattison et al., 2003b; Welle et al., 2003) (Suppl. Table S3). We also compared our findings to other gene expression studies that assessed regenerating and atrophied muscles whose subjects possess similar phenotypes to our experimental groups. Thirty-five percent of the genes were also significantly differentially expressed in normal and regenerating muscles (Fluck et al., 2005; Goetsch et al., 2003; Zhou et al., 2006), while 34% were significantly differentially expressed in normal and atrophying muscles (Batt et al., 2006; Chen et al., 2007; Dapp et al., 2004; Jagoe et al., 2002; Kostrominova et al., 2005; Lecker et al., 2004; Nikawa et al., 2004; Paoni et al., 2002; Raffaello et al., 2006) (Suppl. Table S3). Forty-six percent of the genes that were differentially expressed in the present study have not been reported in previous published microarray data on ageing, atrophy, and regeneration. Although our microarray comparisons with published data were an efficient way to validate results for large numbers of genes, disparities in results might exist between different groups performing similar experiments (Irizarry et al., 2005). Therefore, further verification would be required for affirming the similarities between our analysis and other microarray studies.

3.3. Microarray validation

To validate the microarray findings, we performed qPCR on the 27 genes that had at least a 10-fold change in magnitude between young and old muscles as well as on *Pim1*, the only gene that was up-regulated in old muscle (Table 1). The "housekeeping gene" *HRPT1* was selected as the internal reference because it was expressed at similar levels in young and old muscles (data not shown). In accordance with the microarray data, qPCR results from Gas muscle showed that in all cases, the values were significantly higher for young muscle than for old muscle, except for *Pim1*, which was significantly lower in young muscle (Table 1).

We also located the expression pattern for genes that might be important components in the myogenic signalling pathways of transforming growth factor beta (TGFbeta), Wnt signalling and Notch. To accomplish this, we first verified that the genes in question were differentially expressed in whole muscle tissue of young, adult, and old mice by qPCR (Fig. 4a). Indeed, the genes Fbn1, Aspn, Kera, Cthrc1 and Fmod for TGFbeta signalling, Sfrp2 and Wif1 for Wnt signalling, and Dner for Notch signalling were significantly expressed to a higher level in young whole muscle tissue compared to old thus corroborating the microarray data (Fig. 5a). These results also indicated that there was a gradual loss of expression of the studied genes during the lifetime of the mice. We then delineated the tissue compartment within the muscle

Table 1
QPCR validation of genes from the microarray analysis that were differentially expressed with fold changes greater than 10 between young and old mice as well as *Pim1* (in bold), the only gene that was up-regulated in old muscle. All qPCR fold changes are in agreement with microarray fold changes.

Gene name	Gene abbreviation	Array fold change (Y/O)	QPCR fold change (Y/O)	QPCR *p* value
Extracellular matrix and cell adhesion				
Procollagen, type I, alpha 1	*Col1a1*	168.53	43.5	***
Procollagen, type I, alpha 2	*Col1a2*	167.70	34.3	***
Procollagen, type III, alpha 1	*Col3a1*	38.96	28.9	**
Procollagen, type V, alpha 2	*Col5a2*	21.12	9.7	**
Keratocan	*Kera*	19.94	13.2	***
Secreted acidic cystein rich glycoprotein	*Sparc*	15.99	11.2	***
Procollagen, type VIII, alpha 1	*Col8a1*	14.40	3.9	*
Periostin, osteoblast specific factor	*Postn*	14.01	39.4	***
Procollagen, type VI, alpha 2	*Col6a2*	10.55	4.4	***
Myelination				
Myelin protein zero	*Mpz*	23.30	14.1	***
Myelin basic protein	*Mbp*	13.92	6.1	***
Signal transduction				
Angiotensin receptor-like 1	*Agtrl1*	17.78	7.5	*
Cell cycle/differentiation/development				
RIKEN cDNA 1500015O10 gene	*1500015O10Rik*	44.08	16.8	*
H19 fetal liver mRNA	*H19*	35.97	11.0	***
Tenomodulin	*Tnmd*	19.43	19.6	***
Cyclin-dependent kinase inhibitor 1C (P57)	*Cdkn1c*	14.44	11.8	*
Secreted frizzled-related protein 2	*Sfrp2*	11.24	6.4	***
Follistatin-like 1	*Fstl1*	10.95	6.1	***
Proviral integration site 1	*Pim1*	0.2592	0.2	**
Cytoskeleton				
Myosin, heavy polypeptide 7, cardiac muscle, beta	*Myh7*	87.33	46.9	***
Troponin T1, skeletal, slow	*Tnnt1*	53.45	21.4	***
Myosin, light polypeptide 2, regulatory, cardiac, slow	*Myl2*	48.92	24.7	***
Tropomyosin 3, gamma	*Tpm3*	47.06	7.1	***
Myosin, light polypeptide 3	*Myl3*	24.85	13.3	**
Troponin C, cardiac/slow skeletal	*Tnnc1*	20.91	25.0	***
Myozenin 2	*Myoz2*	14.40	9.0	**
Ankyrin repeat domain 2 (Stretch responsive muscle)	*Ankrd2*	10.36	5.1	***
Unknown				
GTL 2 imprinted maternally expressed untranslated mRNA	*Gtl2*	29.54	13.6	***

[*] *p* between 0.05 and 0.001.
[**] *p* between 0.0009 and 0.0001.
[***] $p < 0.0001$.

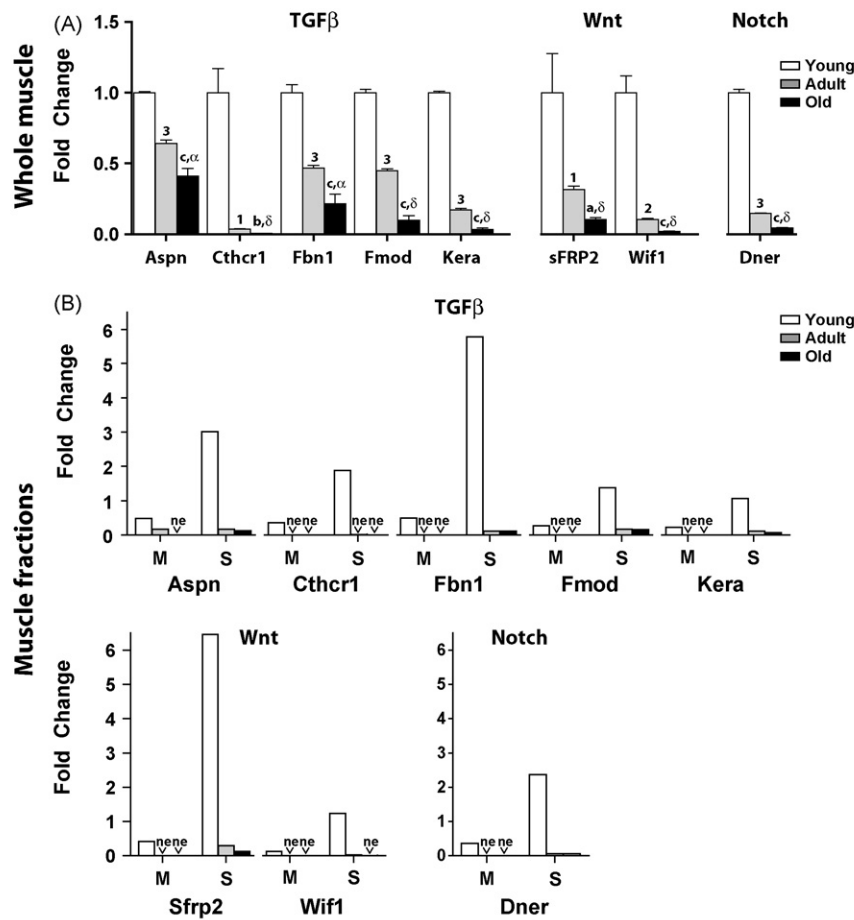

Fig. 5. Expression distribution for genes involved in the myogenic signalling pathways TGFbeta, Wnt and Notch. (a) Graphical representation of gene expression in whole gastrocnemius muscle for the genes Aspn, Cthrc1, Fbn1, Fmod and Kera (TGFbeta signalling); sFRP2 and Wif1 (Wnt signalling); and Dner (Notch signalling) in young, adult, and old mice ($n = 3$). The comparison between aged muscles were denoted as follows: young versus adult (1, 2, and 3); young versus old (a, b, and c); adult versus old (α, β, and δ). $p < 0.05$ (1, a, and α), $p < 0.01$ (2, b, and β) and $p < 0.001$ (3, c, and δ). (b) Graphical representation of gene expression in pooled ($n = 4$) myogenic (M) and stromal (S) gastrocnemius muscle fractions for the genes Aspn, Cthrc1, Fbn1, Fmod and Kera (TGFbeta signalling); sFRP2 and Wif1 (Wnt signalling); and Dner (Notch signalling) in young, adult, and old mice. ("ne" denotes not expressed.)

that might express the genes of interest by separating the myogenic fraction represented by gastrocnemius fibres (containing quiescent satellite cells and myofibres) from the stromal fraction (containing all other cell types including some activated satellite cells) of young, adult and old muscle (Fig. 5b). Our results show higher expression of the genes in question occurring in the stromal fraction compared to the myogenic fraction in young mice and minimal expression of the genes in adult and old mice regardless of the muscle fraction (Fig. 5b). This result was expected as some cells comprising the stromal fraction would be integral to the proper maintenance of the niche. To fine tune the expression pattern, different cell types comprising the muscle tissue, that included proliferating primary MPCs (myoblasts) and stromal cells were isolated and grown *in vitro*. However, their assessment was unclear as *in vitro* culture effects and variable isolation efficiencies resulted in uneven growth and differentiation rates not withstanding if the isolate was from young, adult or old muscles supporting the importance of muscle tissue microenvironment for proper gene expression (Suppl. Fig. S1). Taken together these results imply that key components involved in TGFbeta, Wnt and Notch signalling are derived from accessory non-myogenic cells represented by the stromal fraction.

We also analyzed the expression pattern of the genes that might be important components in the myogenic signalling pathways of transforming growth factor beta (TGFbeta), Wnt signalling and Notch in the context of muscle regeneration (Fig. 6). Regeneration in muscle was induced by cardiotoxin (CTX) injection. qPCR analyses of CTX-injured compared to saline control muscles, revealed that all the genes of interest were highly expressed during muscle regeneration, that is during satellite cell activation and proliferation (Fig. 6). The high expression levels tended to recede over time, to levels closer to controls. Hence these results show that during regeneration the genes of interest are highly expressed suggesting that they might have a role in satellite cell integrity and their loss of expression in old muscle might compound the observed regenerative defects.

In addition we performed western blot analyses in young and old gastrocnemius muscles of the genes above (Suppl. Fig. S2). The results indicate only modest significant differences for four genes; SFRP2 and Fmod (lower in old compared to young) and Cthrc and Wif1 (higher in old compared to young). Moreover, three genes, Aspn, Dner and Kera, had no significant changes (Suppl. Fig. S2). Hence, the RNA expression data, despite verification by qPCR, does not correspond completely to the protein expression data. There are many reasons for this anomaly that include nuclear export and mRNA localization, transcript stability, translational regulation and protein degradation. Other factors potentially might affect protein stability or *in vivo* half life such as post-translational modifications for example, glycosylation and phosphorylation, and proteolytic cleavage. The control mechanisms for any of these systems might be altered or perhaps compromised to purposely maintain

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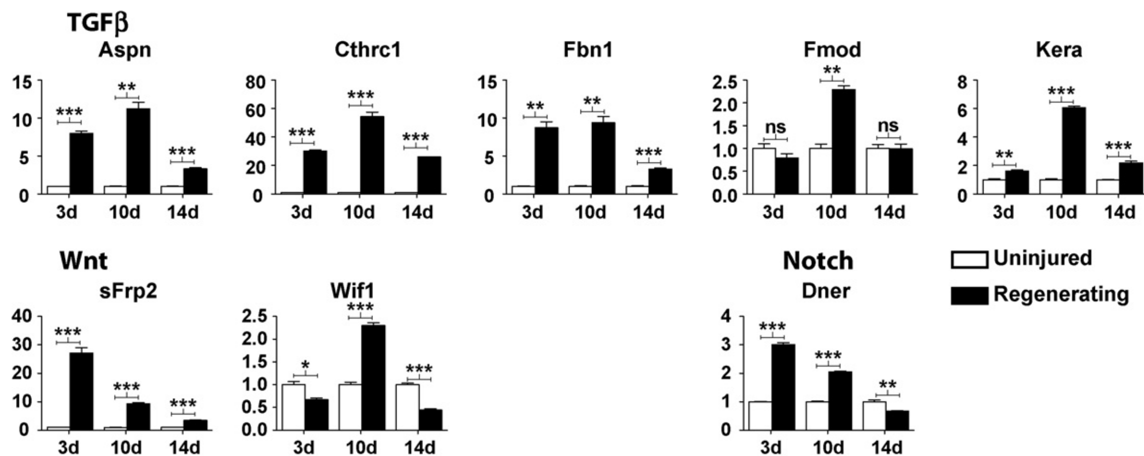

Fig. 6. Expression distribution for genes involved in the myogenic signalling pathways TGFbeta, Wnt and Notch during muscle regeneration. Graphical representation of gene expression in gastrocnemius muscle 3, 10 and 14 days post-saline (uninjured) or cardiotoxin (regenerating) injection for the genes Aspn, Cthrc1, Fbn1, Fmod and Kera (TGFbeta signalling); sFRP2 and Wif1 (Wnt signalling); and Dner (Notch signalling). For saline ($n = 4$) and cardiotoxin ($n = 4$) (*p = 0.05–0.001; **p = 0.0009–0.0001; ***$p < 0.0001$).

higher protein levels in the aged mice. This calls upon further research for assessing protein function to focus on specific experiments designed for certain pathways and on specific cell types that are important to the muscle and specifically to its niche.

4. Discussion

High-density oligonucleotide microarrays are a valuable tool for studying differences in transcriptional expression on a genome-wide scale. Microarray analyses of mouse, rat, and human muscles have been used to study expression differences in ageing muscle (Edwards et al., 2007; Lee et al., 1999; Pattison et al., 2003a,b; Welle et al., 2003), as well as related studies concerning muscle atrophy (Batt et al., 2006; Chen et al., 2007; Dapp et al., 2004; Jagoe et al., 2002; Kostrominova et al., 2005; Lecker et al., 2004; Nikawa et al., 2004; Paoni et al., 2002; Raffaello et al., 2006), and regeneration after injury (Fluck et al., 2005; Goetsch et al., 2003; Zhou et al., 2006). These studies have reported major differences in the expression of genes involved in DNA damage repair, stress response, reinnervation, immune/inflammatory responses, RNA binding and splicing, and proteasome degradation.

Adult muscle is a stable tissue where degeneration/regeneration of muscle fibres does not readily occur (Shi and Garry, 2006). Consequently, the slow rate of fibre and satellite cell turnover in adult muscle tends to preclude the identification of any real differences in the growth and differentiation of stem cells that are required to maintain the integrity of old muscle tissue in the face of mounting degeneration/regeneration. To circumvent this shortcoming, we used young sexually immature 3-week-old mice as our control group in our genome-wide expression analysis. Young mice represent a postnatal stage of muscle growth and development that is distinct from adult and old mice. Muscle in neonates undergoes rapid development, with efficient satellite cell activation, proliferation, and differentiation programs (Shi and Garry, 2006). For transcriptional profiling purposes, young mice represent a normal physiological phase of muscle development for highlighting differences concerning regeneration defects with old muscle unlike data obtained after the induction of massive injury (Goetsch et al., 2003). Hence, comparisons of the gene expression profiles of young and old muscles should target significant differences with respect to the deficit in regeneration components of old muscle in a physiological setting.

Two findings regarding our analysis are most apparent. First, our results reveal that old skeletal muscle does not have a deficit in quiescent satellite cells and fusion competent cells when compared to the young growing muscles. However, it has a severe paucity of activated and proliferating MPCs which may affect the quality of regeneration in aged mice. Indeed, our data suggests that MPCs from old muscle undergo precocious differentiation by preferentially fusing rather than remain activated and proliferative. Second, our data also suggest that some cells comprising the stromal fraction of the skeletal muscle milieu are integral to proper maintenance and function. This underscores the contribution of non-myogenic cells in affecting the gene expression differences between young and old mice.

Some of the differences that we observed when comparing old to young muscle were expected. Indeed, the significantly increased expression of genes involved in myelination, the cytoskeleton, and protein translation in young muscle reflects the growth and development of the skeletal muscle that is opposite to age-related atrophic defects in old muscle. Significant differences in factors required to mediate satellite cell proliferation and differentiation were also brought to light in our screen.

A noteworthy result of the present study was the large number of ECM and adhesion genes that were expressed at significantly higher levels in young mice. The ECM surrounding myofibres is a dynamic extracellular network made up of an assortment of molecules that cooperate with each other and with the sarcolemma, cytoskeleton, and nuclear proteins to maintain skeletal muscle integrity and tissue architecture. For the satellite cell, the ECM and adhesion proteins are components of a microenvironment or niche, essential for how the cell responds to local and systemic cues. The ECM is critical in influencing satellite cell functioning through its interactions with cytokines, growth factors, and other cells to bring about the proper events required for satellite cell activation, proliferation, and differentiation (Gopinath and Rando, 2008). Therefore, the robust up-regulation of ECM genes in young muscle might provide an environment permitting efficient satellite cell functioning that is remiss in old muscle. For example, one of these genes, *Procollagen type VI alpha 2* (*Col6a2*), is mutated in both Bethlem myopathy and Ullrich congenital muscular dystrophy, two disorders characterized by severe muscle shortening and weakness (Lampe and Bushby, 2005). Col6a2 is a subunit chain that, together with Col6a1, whose gene was also found in our screen, and Col6a3 make up Type VI collagen filaments, an integral component of the basal lamina ECM

of muscle (Kuo et al., 1997). In muscles from dystrophic patients, collagen VI labelling is reduced and discontinuous at the basal lamina of muscle fibres where quiescent satellite cells reside (Merlini et al., 2008). The specific loss of collagen VI may also influence binding to other cell-surface receptors, integrins (Tulla et al., 2001), and NG2 (Tillet et al., 1997), or interactions with other ECM components, including collagen IV (Kuo et al., 1997), fibronectin (Sabatelli et al., 2001), biglycan, decorin (Wiberg et al., 2001), and microfibril-associated glycoprotein-1 (MAGP-1) (Finnis and Gibson, 1997).

Another ECM protein that has been recently described to play an essential role in maintaining the niche and promoting muscle regeneration is Secreted Protein Acidic and Rich in Cysteine (Sparc), also known as osteonectin and BM-40, which we found to be significantly up-regulated in young muscle. Sparc is a multifunctional 43-kDa protein that specifically binds several ECM collagens involved in modulating cell–ECM interactions by participating in organizing the basal lamina (Bradshaw and Sage, 2001). Sparc gene expression increases during differentiation in myoblast cell lines and its inhibition prevents differentiation (Cho et al., 2000; Motamed et al., 2003). Gene expression profiles of Duchenne muscular dystrophy and alpha-sarcoglycan deficiencies, where muscle tissue is undergoing vigorous regeneration, also show that *Sparc* expression is significantly up-regulated (Chen et al., 2000; Haslett et al., 2002; Noguchi et al., 2003). Confirming the importance of *Sparc* are studies that show its up-regulation during muscle development and in regenerating muscle caused by disease and following injury (Ferre et al., 2007; Jørgensen et al., 2009).

Our results suggest that an inability to block TGFbeta activity is an important failing of old muscle niche. Indeed, Carlson et al. (2008a) recently showed that TGFbeta plays a role in an ageing pathway through its induction of pSmad3. Old muscle produces excessive TGFbeta, which interferes with the regenerative capacity of resident satellite cells by blocking their proliferation (Carlson et al., 2008b). The attenuation of TGFbeta in old injured muscle restores the regenerative capacity of satellite cells in vivo (Carlson et al., 2008a). Our findings suggest that the ECM gene *Fibrillin 1* (*Fbn1*), which is significantly more highly expressed in young muscle, might play a central role in maintaining an efficient satellite cell niche counteracting the effects of TGFbeta. Indeed, Fbn1 has been shown to negatively regulate TGFbeta activation and signalling through its association with the latent TGFbeta binding protein (Neptune et al., 2003). The importance of Fbn1 to satellite cell niche integrity has been corroborated by other studies showing higher levels in young and regenerating muscle expression profiles and lower levels in atrophic muscle profiles. *Fbn1* mutations are associated with Marfan syndrome, which is generally regarded as a connective tissue disorder. However, recent studies have described individuals with *Fbn1* mutations who are unable to increase muscle mass in response to growth and exercise, also have severe muscle weakness and profound muscle hypoplasia (Behan et al., 2003; Percheron et al., 2007). *Fbn1* deficiency results in excess TGFbeta activity, which impairs satellite cell proliferation and differentiation. For example, *Fbn1*-deficient mice have increased TGFbeta activity and are unable to regenerate muscle (Cohn et al., 2007). On the other hand, antagonizing TGFbeta signalling normalizes muscle architecture, repair, and function *in vivo* (Cohn et al., 2007). Other genes that were found significantly expressed higher in young animals in our screen might also be involved in counteracting TGFbeta. These include collagen triple helix repeat containing 1 (Cthrc1), which has been shown to repress TGFbeta signalling in smooth muscle cells (LeClair et al., 2007), Asporin (Aspn), which binds to and inactivates TGFbeta in chondrocytic cells (Nakajima et al., 2007), Keratocan (Kera), which blocks TGFbeta activity in keratocytes (Kawakita et al., 2005), and Fibromodulin (Fmod), a proteoglycan that binds TGFbeta in vitro (Hildebrand et al., 1994).

Another critical finding of our gene expression screen are the up-regulated genes involved in the Notch and Wnt pathways whose control is thought to be necessary for normal adult myogenesis (Carlson et al., 2008b). In this case two Wnt antagonists, *Secreted frizzled-related protein 2* (*Sfrp2*) and *Wnt inhibitory factor 1* (*Wif1*), and *Delta/Notch-like EGF related receptor* (*Dner*) were up-regulated in young muscle.

Wnt signalling is mediated by a family of 19 glycoproteins that control gene expression, cell fate determination, and the survival of different tissue types during embryogenesis and postnatal growth (Nusse, 2005). In embryogenesis, Wnt is required for muscle formation (Anakwe et al., 2003; Cossu and Borello, 1999) and for myogenic differentiation in cell lines (Petropoulos and Skerjanc, 2002). In the Adult, a recent report has described the temporal role of Wnt activity in controlling proliferation and differentiation of satellite cells (Brack et al., 2008). The addition of Wnt during the early stages of myogenesis causes inappropriately timed cell differentiation, while the inhibition of Wnt at later stages results in decreased fibre formation (Brack et al., 2008). Influencing Wnt activity by blocking it with the inhibitors Sfrp2 and Wif1 may thus be crucial in promoting efficient myoblast proliferation, which is absent in old muscle. Indeed, the importance of inhibiting Wnt activity is highlighted in ageing muscle where Brack et al. (2007) have shown that increased Wnt signalling is associated with regenerative impairment and increased tissue fibrosis. A finding that is corroborated by reports that describe accelerated ageing, dysfunction in stem cells and fibrosis in various tissues is associated with increased Wnt activity caused by the targeted deletion of Klotho, a secreted protein that binds and sequesters Wnt (Liu et al., 2007).

Contrary to the function of Wnts in myogenesis, the age-related decline of the regenerative potential is linked to a falling off in Notch signalling that can be reversed by activating the Notch pathway in muscle (Carlson et al., 2008a; Conboy et al., 2003). In this regard, the discovery of the Notch ligand/receptor Dner, a neuron specific transmembrane protein that we found up-regulated in young muscle, might be critical. We did not observe significant differences in *Dner* expression in other ageing, atrophy, or regeneration transcriptional profiles, suggesting that it plays an important role in regeneration following the physiological degeneration that occurs in old muscle. The implication of Notch activation is emphasized by the increase in the activated form of Notch and increased levels of Notch ligand, Delta, within 24 h after a muscle injury (Conboy and Rando, 2002). Activated Notch is required to elevate the satellite cell progeny by rapidly increasing cell cycle, which results in the generation of very large numbers of progenitor cells. Inhibition of Notch signalling during this early phase of satellite cell activation inhibits effective muscle regeneration by preventing this expansion. On the other hand, a reduction in Notch signalling is required for progenitor cells to proceed to the next step in becoming fusion competent myoblasts (Conboy and Rando, 2002). A recent report highlighted the role of Dner as a Notch ligand on the myoblast cell line C2C12 that endogenously expresses Notch and its downstream signalling molecules (Eiraku et al., 2005).

The “free radical theory of ageing” posits that functional and structural damage to skeletal muscle cells over time results from oxidative damage caused by reactive oxygen species (ROS) (Balaban et al., 2005; de Magalhaes and Church, 2006; Harman, 1956). Studies have shown that oxidative stress affects proper primary satellite cell functioning (Renault et al., 2000). Mitochondria are the primary source of ROS, which result from ATP production. Indeed, adaptations of antioxidant defence systems

are crucial to deal with the increased generation of reactive oxygen species (ROS) during muscle regeneration in exercise and injury. Young developing muscles have adaptive mechanisms to defend themselves efficiently. However, it is not known how or why these antioxidant mechanisms break down during ageing (Yu and Chung, 2006). Our screen unveiled that certain metabolic factors involved in reducing oxidative stress are more highly expressed in young muscle. One of these is nicotinamide nucleotide transhydrogenase (Nnt), a mitochondrial proton translocase that transfers protons from NADH to $NADP^+$ to produce NADPH (Rydstrom, 2006). NADPH is essential in maintaining reduced glutathione (GSH), which participates in the defence against ROS. Longevity in the nematode *C. elegans* is associated with mitochondrial stability and the targeted deletion of Nnt increases sensitivity to oxidative stress (Arkblad et al., 2005; Lee et al., 2003). In addition, Nnt influences the longevity of mice that are deficient in mitochondrial superoxide dismutase, a ROS stabilizing enzyme (Huang et al., 2006).

The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was also up-regulated in young muscle and may play a role as a sensor of oxidative stress. *GAPDH* is generally regarded as a housekeeping gene involved in energy metabolism, but several non-traditional activities have also been assigned to it (Chuang et al., 2005). The conformation and function of GAPDH is altered by oxidative stress in vitro and in vivo due to the highly reactive cysteines in its active site (Pierce et al., 2006, 2007; Yasuda et al., 1998). Oxidative stress caused structural alterations in GAPDH and leads to the inhibition of its activity. In a mouse model of skeletal muscle regeneration, GAPDH is structurally and functionally altered by oxidative stress that is predominately caused by infiltrating inflammatory cells, that has been observed during ageing (Pierce et al., 2007). Under this oxidative stress, inoperative GAPDH redirects glucose metabolism through the pentose phosphate pathway, which produces the NADPH required for maintaining reduced GSH. It also uncouples glucose metabolism from the production of ATP and oxidative intermediates (Albina et al., 1999).

The results of the present study underscored the importance of factors involved in maintaining satellite cell function during the ageing process. We used a novel experimental design to discriminate differentially expressed genes that are involved in the skeletal muscle breakdown and repair that occurs during ageing. By comparing young growing muscle to old degenerating/regenerating muscle, our expression profile detected genes essential for the proper functioning of satellite cells. Indeed, in old muscles, we found reduced expression of genes involved in cellular processes and signalling networks committed to satellite cell activation, proliferation, and differentiation. The study of these factors and their corresponding pathways will provide a better understanding of the role of satellite cells in myofibre maintenance during the ageing process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2009.11.001.

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