Stem Cell Research (2011) 7, 112–123

available at www.sciencedirect.com

www.elsevier.com/locate/scr

REGULAR ARTICLES

Alpha 6 integrin is important for myogenic stem cell differentiation

Karlijn J. Wilschut [a,*], Helena T.A. van Tol [a], Ger J.A. Arkesteijn [b], Henk P. Haagsman [b], Bernard A.J. Roelen [a]

[a] *Department of Farm Animal Health, Faculty of Veterinary Medicine, Yalelaan 104, 3584 CM, Utrecht University, Utrecht, The Netherlands*
[b] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

Received 7 December 2010; received in revised form 17 April 2011; accepted 2 May 2011
Available online 10 May 2011

Abstract A muscle progenitor cell population, other than muscle satellite cells, can be isolated and purified from porcine muscle tissue. We show the presence of at least two types of stem cells in porcine muscle: those that express α6 integrin and those that lack expression of this integrin type. By flow cytometry, we could select for myogenic stem cell populations expressing the neural cell adhesion molecule in the presence and absence of α6 integrin. The expression of α6 integrin showed an advantage in the formation of myotubes, possibly by an improved cell fusion capacity. This notion was strengthened by qRT-PCR analysis showing sustained *PAX7*, *MYF5* and *DESMIN* expression and a strong myogenic differentiation capacity of this stem cell population. Selective inhibition of α6 integrin function, both by blocking antibodies and RNA interference, showed the importance of α6 integrin in myogenic differentiation of muscle stem cells. It is concluded that α6 integrin expression can be used as biomarker to select for highly myogenic cell populations in muscle tissue.

1. Introduction

Skeletal muscle contains a population of stem cells to support postnatal muscle growth and regeneration. These cells, characterized as muscle satellite cells, adopt a position between the basal lamina and plasma membrane of muscle fibers and possess a self-renewing capacity to sustain the resident stem cell pool (Montarras et al., 2005). The characteristic PAX7 expression by satellite cells is important to maintain postnatal self-renewal and myogenic commitment until activation upon damage or during development occurs (Seale et al., 2000; McKinnell et al., 2008; Relaix et al., 2005; Oustanina et al., 2004). Here, cells start to proliferate and differentiate into myoblasts, a process coordinated by the muscle regulatory factors (MRFs) Myf5 and MyoD (Buckingham et al., 2003; Rudnicki et al., 1993; Tajbakhsh, 2005). The second differentiation step occurs after myoblast alignment, initiating myoblast fusion resulting in the formation of myotubes. The MRF Myogenin controls

* Corresponding author at: Cardiovascular Research Institute, Department of Pediatrics, University of California San Francisco, 513 Parnassus Avenue, Health Sciences East 1401, San Francisco, CA 94143, USA. Fax: +1 415 514 0235.
E-mail addresses: karlijn.wilschut@ucsf.edu (K.J. Wilschut), h.t.a.tol@uu.nl (H.T.A. van Tol), g.j.a.arkesteijn@uu.nl (G.J.A. Arkesteijn), h.p.haagsman@uu.nl (H.P. Haagsman), b.a.j.roelen@uu.nl (B.A.J. Roelen).

1873-5061/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.scr.2011.05.001

terminal differentiation into mature myofibers (Pownall et al., 2002; Alapat et al., 2009).

Integrins function as receptors important for adhesion to extracellular matrix (ECM) proteins or to other cells. They are composed of α- (18 types) and β- (8 types) subunits that can form 24 distinct heterodimers of which the composition dictates ligand specificity (Hemler, 1999; Hynes, 2002; van der Flier and Sonnenberg, 2001). In particular the integrin β1 has a role in regulating muscle integrity and is essential for myoblast adhesion to the ECM (Menko and Boettiger, 1987). During muscle development various integrin subunits are expressed including α1, α4, α5, α6, α7, αV, β1 and β3. They play roles in mediating modulation of cell proliferation, differentiation, migration, polarity, and motility triggering calcium influx and are involved in apoptosis (Hynes, 2002; Danen and Sonnenberg, 2003; Gullberg et al., 1998). The integrin α7β1 is expressed on myoblasts localized in and around the myotendinous and neuromuscular junction of the muscle fiber and is required for muscle maintenance (Bao et al., 1993; Hayashi et al., 1998). In a study with quail embryonic muscle, α5 integrin in myoblasts was demonstrated to stimulate proliferation, whereas α6 integrin mediated differentiation (Sastry et al., 1996). This suggests distinct functions for α-integrins in regulating myogenesis from proliferation towards terminal differentiation.

Functions of α6 integrin (α6ITG) have been studied using a GoH3 neutralizing antibody directed against α6ITG. It is suggested that α6β1 is involved in adhesion to the E8-cell-binding site of laminin in non-muscle cells, whereas in muscle cells, not α6β1, but a different β1 integrin-series binds laminin (von der Mark et al., 1991; Sonnenberg et al., 1990). Alpha 6 integrin is expressed during early mouse development at the stages of laminin containing basement formation, which remains during embryogenesis (Hierck et al., 1993). Here, delamination of Myf5 expressing muscle progenitor cells formed laminin-rich myotome mediated by α6β1 integrin expression (Bajanca et al., 2004, 2006). Previous results showed that sustained α6ITG expression of muscle stem cells cultured on Matrigel surface coating was correlated with a high myogenic differentiation in vitro, suggesting a role for α6ITG during muscle stem cell differentiation (Wilschut et al., 2010).

Muscle tissue contains a heterogeneous population of muscle progenitor cells (Zammit et al., 2006; Kuang et al., 2007; Cerletti et al., 2008). Selection for specific progenitor cells can be important to enrich for homogeneous cell populations to study function and differentiation potentials. Indeed, in several studies integrins as well as other membrane associated biomarkers were used to select for specific stem cell populations (Cerletti et al., 2008; Webster et al., 1988; Sherwood et al., 2004; Tamaki et al., 2002; Zheng et al., 2007). In this respect, expression of the neuronal cell adhesion molecule (NCAM) by satellite cells has been used to mark a myogenic cell population (Capkovic et al., 2008; Mesires and Doumit, 2002).

In this study, muscle stem cells isolated from the pig were used. We used flow cytometry to sort for muscle stem cells based on biomarker expression of NCAM and α6ITG. These cells were examined for their myogenic differentiation capacity revealing a potential function of α6ITG during myogenesis.

2. Results

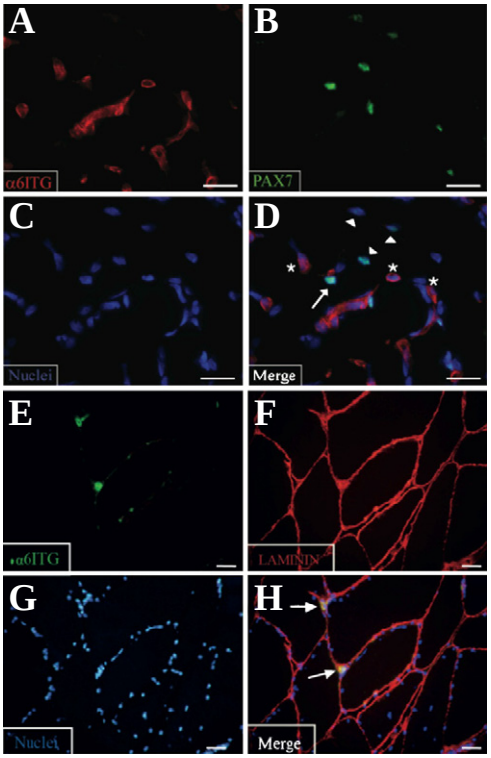
2.1. Muscle tissue contains satellite cells with distinct α6ITG expression levels

To specifically localize cells expressing α6ITG and to detect muscle satellite cells by PAX7 expression, cryosections of porcine semitendinosus muscle were stained with antibodies against PAX7, α6ITG and Laminin. Two types of satellite cells could be distinguished; (1) PAX7-expressing (satellite) cells that lacked α6ITG expression (Fig. 1D; arrowhead) and (2) PAX7-expressing cells (satellite) that co-expressed α6ITG (Fig. 1D; arrow). Additionally, α6ITG expressing cells that lacked PAX7 expression were observed (Fig. 1D; asterisks). Thus, the identification of at least two different satellite cell types indicates the heterogeneity of satellite cells in muscle. Furthermore, α6ITG expressing cells were observed adjacent to the extracellular matrix component laminin, indicating the presence of these cells in laminin rich muscle areas (Fig. 1E-H).

2.2. Selection for α6ITG expression results in highly myogenic committed stem cell population

Isolated porcine primary muscle stem cells were expanded on Matrigel-coated flasks. Myogenic stem cell populations were selected by flow cytometry based on antibodies directed against NCAM (expressed by satellite cells) and α6ITG. The FITC- and PE-conjugated isotype control antibodies were used to gate the background. Three cell populations were sorted individually (Fig. 2); (1) NCAM$^+$/ α6ITG$^+$ population (22%), (2) NCAM$^+$/ α6ITG$^-$ cells (8%) and (3) NCAM$^-$/ α6ITG$^-$ cells (3%) (double negative for α6ITG and NCAM), most likely representing non-myogenic cells.

All other experiments were conducted using laminin- and Matrigel-coated plates functioning as substrates containing α6ITG engaging ligands (Sonnenberg et al., 1990). The myogenic differentiation capacity of the sorted NCAM$^+$/ α6ITG$^+$ cell population was demonstrated by detection of myogenic related protein expression. Detection of MYOGENIN, DESMIN and MYHC expression (Fig. 3A) revealed the high myogenic character of this cell population and was also indicated by formation of (striated) myotubes after 4 days of differentiation (Fig. 3A, arrow and arrowhead). The expression of α6ITG confirmed cell sorting efficiency. Cells expressing α6ITG formed large multinucleated myotubes while cells lacking α6ITG expression only displayed alignment of the cells without fusion (Fig. 3B). Myotube formation was never observed in NCAM$^+$ cells lacking α6ITG expression (data not shown). Gene expression was quantified by qRT-PCR and mRNA levels were determined relative to those of unsorted primary muscle cells. The NCAM$^+$/ α6ITG$^+$ population showed a significantly ($p<0.001$) higher expression of *A6ITG* mRNA compared to the expression of the NCAM$^+$/ α6ITG$^-$ sorted population demonstrating successful separation of the populations (Fig. 3C). Interestingly, the NCAM$^+$/ α6ITG$^-$ population expressed significantly higher levels of *A7ITG* mRNA, whereas, the NCAM$^-$/ α6ITG$^-$ population, expressed significantly ($p<0.001$) higher levels of *A5ITG* mRNA. The *B1ITG* mRNA levels were similar between the two NCAM$^+$ populations.



I

Cell type	#	Percentage
α6ITG+ cells	140	41%
PAX7+ cells	41	12%
α6ITG+/ PAX7+	24	7%
α6ITG-/ PAX7+	18	5%
Nuclei	*344*	*100%*

Figure 1 Immunofluorescence of PAX7 and α6ITG expression in transverse cryosections of semitendinosus muscle tissue. **A.** Localization of α6ITG expression with anti-α6ITG antibodies (GoH3, red). **B.** Satellite cells are identified by PAX7 expression using anti-PAX7 antibodies (PAX7, green). **C, G.** Visualization of nuclei by DAPI (blue). **D.** Merged channels showing three different cell populations; α6ITG expressing satellite cells (arrows), satellite cells lacking α6ITG expression (arrowheads) and unidentified muscle cells with α6ITG expression (asterisks). Scale bars represent 20 μm. **E.** Localization of α6ITG expression with anti-α6ITG antibodies (GoH3, green). **F.** Visualization of the extracellular matrix component laminin (red). **H.** Merged channels identifying the sub-laminar position of α6ITG positive cells. I. Determination of frequency of different cell types. Scale bars represent 50 μm.

Examination of muscle related gene expression revealed that both NCAM$^+$ populations comprised a high *PAX7* expression with a significantly ($p<0.05$) higher *MYF5* expression in α6ITG$^+$ cells (Fig. 3C). MYF5 is important for initiation of muscle differentiation, suggesting a stronger myogenic potential of the α6ITG$^+$ cells. However, *DESMIN* expression was significantly ($p<0.05$) higher in α6ITG$^-$ cells, indicating a strong myogenic feature despite the lower *MYF5* expression. The NCAM$^-$/ α6ITG$^-$ cell population showed lower expression levels of *MYF5* and *DESMIN*, which are important during myogenesis, demonstrating the non-myogenic feature of these cells. Morphology of both NCAM$^+$ populations showed

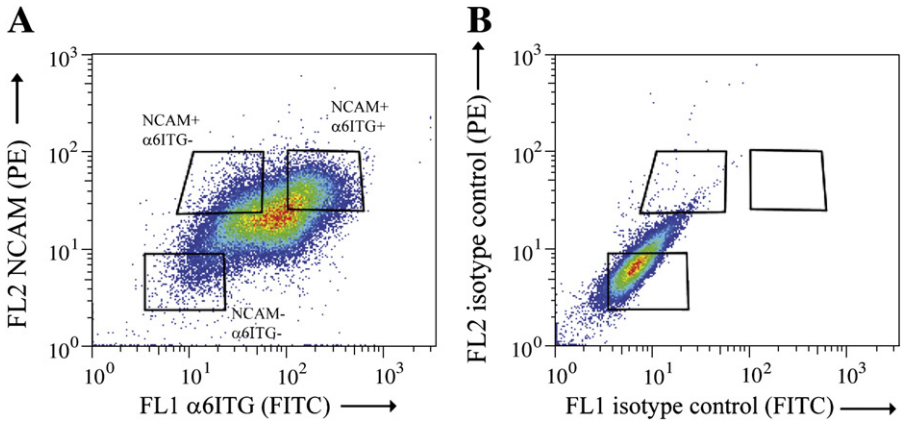


Figure 2 Flow cytometric analysis of primary muscle stem cells for NCAM and α6ITG expression. **A.** Gates represent sorted cell populations corresponding to specific antibody staining as indicated. NCAM$^+$ cells were visualized by the PE-channel and the α6ITG$^+$ cells were visualized in FITC-channel. The NCAM$^+$/ α6ITG$^+$ population represented 22%, NCAM$^+$/ α6ITG$^-$ cells 8% and NCAM$^-$/ α6ITG$^-$ 3% of the total population. **B.** Negative isotype control represents non-specific background staining.

bi- and triangular shaped cells with several pseudopodia. However, α6ITG$^+$ cells exhibiting a more flattened morphology compared to the α6ITG$^-$ cells (Fig. 4A).

The difference between the two populations (NCAM$^+$/ α6ITG$^{+/-}$) concerning their growth rate was monitored by the determination of cell numbers. Cells lacking α6ITG expression had a 3-fold higher expansion rate compared to α6ITG$^+$ cells as indicated at day 7 of proliferation (Fig. 4B). In differentiation medium, NCAM$^+$/ α6ITG$^-$ cells sustained a high proliferative activity suggesting no initiation of differentiation.

2.3. Alpha 6 integrin is required for robust myogenic differentiation

To examine and compare the myogenic differentiation capacity of the NCAM$^+$/ α6ITG$^{+/-}$ cell populations the expression of genes involved in myogenesis was measured by qRT-PCR during differentiation. Here, both NCAM$^+$/ α6ITG$^+$ and NCAM$^+$/ α6ITG$^-$populations showed approximately equal *PAX7* mRNA levels directly after sorting. Initiation of differentiation resulted in a drop of *PAX7* expression in both populations, whereas *PAX7* expression recovered in α6ITG$^+$ cells significantly towards α6ITG$^-$ cells during differentiation (Fig. 5A). Levels of *MYF5* mRNA, coding for the transcriptional initiator of muscle stem cell differentiation, remained significantly higher ($p<0.01$) in α6ITG$^+$ cells compared to α6ITG$^-$ cells after sorting. This indicates the low myogenic orientation of these cells when lacking α6ITG expression (Fig. 5B). In the α6ITG$^-$ cells a decrease in *DESMIN* expression was observed (Fig. 5C). The expression of intermediated muscle filament protein *DESMIN* sustained in α6ITG$^+$ cells ($p<0.001$), while *DESMIN* in α6ITG$^-$ cells reduced considerably after several days of differentiation. Furthermore, the morphological myotube formation by α6ITG$^+$ cells was correlated to the remarkable increase of *MYHC* expression (component of the fast type muscle fiber compartment) (Fig. 5D). This resulted in a significant difference in *MYHC* expression between the populations at day 4 of differentiation as supported morphologically (data not shown). Furthermore, the α6ITG expression determined over time during 4 days of differentiation showed that both cell populations undergo an increase in α6ITG expression at the time point of differentiation (Fig. 5E). During differentiation, the cells depleted for α6ITG show a decrease in expression, whereas a sustained increased gene expression is observed in the α6ITG+cells.

2.4. Blocking of α6ITG inhibits myogenic differentiation of primary muscle stem cells

Primary muscle stem cells were cultured on laminin-coated cover slips. The α6ITG function was blocked with anti-α6ITG antibody (GoH3) incubation for 7 days (Sonnenberg et al., 1988). The antibody binding to the cells was visualized by immunofluorescence (Fig. 6A). Blocking of α6ITG function did not interfere with the adhesion of myogenic cells to the cover slips and myogenic cell division. This was indicated by the expression of DESMIN both in the presence or absence of the blocking antibodies (Fig. 6B). The negative staining of isotype control antibodies indicated antibody specificity (Fig. 6C). Blocking of the α6ITG function by antibodies resulted in the inhibition of primary muscle stem cell fusion into myotubes after exposure to differentiation conditions for 6 days. Cells lacking GoH3 antibody treatment were able to form myotubes suggesting an important function of α6ITG during myogenic differentiation (Fig. 6D).

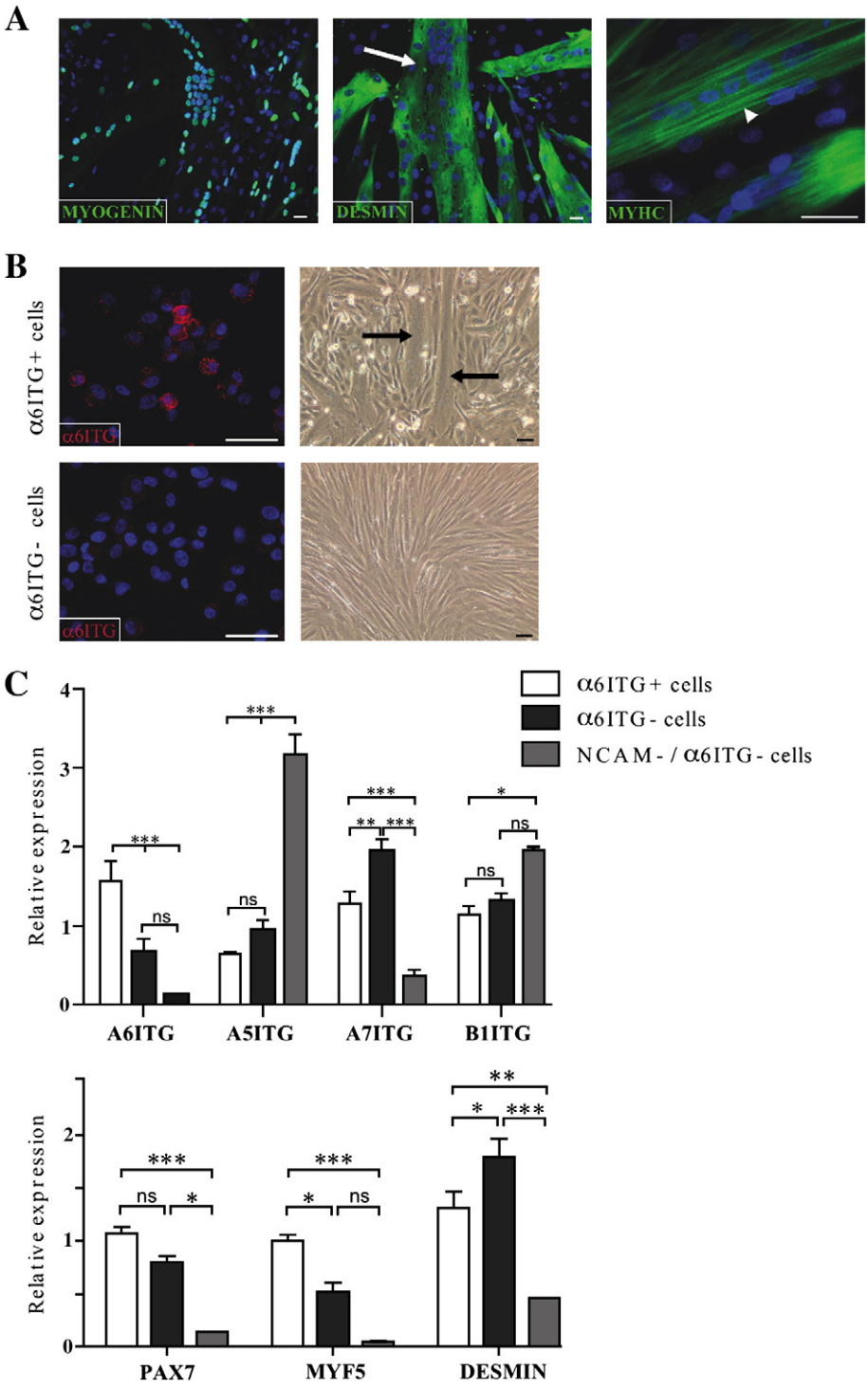
2.5. RNAi mediated knockdown of α6ITG results in inhibition of differentiation

The role of α6ITG in muscle stem cell differentiation was further examined by transient downregulation using siRNA in proliferating primary muscle stem cells. Transfection with siRNA targeted against α6ITG expression resulted in significant decreased expression of 90% after 24 h (Fig. 7A; $P<0.01$). However, no significance is observed after 48 h post transfection. Control siRNA (mock) did not result in a significant downregulation of α6ITG expression. Protein detection using immunoblotting indicated the downregulation of α6ITG till 72 h compared to mock control (Fig. 7B). MYOGENIN expression was not detected during the first 96 h in both groups indicating the proliferative stage of the cells. After 96 h MYOGENIN expression was upregulated in the mock control group, but remained low in cells with downregulated α6ITG expression suggesting inhibition of terminal myogenic differentiation in these cells. Indeed, cells with downregulated α6ITG expression only formed a few thin myotubes, even after prolonged time, whereas large myotubes were observed in the mock control group (Fig. 7D). In addition, the expression of *MYHC* as detected with qRT-PCR was significantly decreased after α6ITG downregulation (Fig. 7C). Expression levels of MYF5, MYOD and DESMIN were not significantly different between the cells with downregulated α6ITG and mock control cells (data not shown).

3. Discussion

In muscle tissue, satellite cells are heterogeneous as demonstrated by a distinction in α6ITG expression (Fig. 1). Laminin is expressed along the muscle fiber within the extracellular matrix enclosing the muscle fiber, where the laminin ligands cause α6/β1 integrin heterodimers receptor binding (Sonnenberg et al., 1990; Hall et al., 1990; Aumailley et al., 1990; Le Bellego et al., 2002). The localization of α6ITG integrin expressing satellite cells with laminin-rich extracellular matrix enclosing the muscle fiber emphasizes this important ligand binding interaction (Fig. 1H). Previously, we observed that increased levels of α6ITG expression in primary muscle stem cells were correlated with a better myogenic differentiation capacity (Wilschut et al., 2010). Therefore, we further investigated the role of α6ITG during myogenesis by selecting and sorting for NCAM expressing cells with or without α6ITG expression from isolated primary muscle stem cell culture. NCAM is involved in myoblast fusion and is expressed by satellite cells that are associated with myogenic cell commitment (Capkovic et al., 2008; Charlton et al., 2000). Thereby we could distinguish between myogenic and non-myogenic cells (Webster et al., 1988; Capkovic et al., 2008; Mesires and Doumit, 2002; Blanton et al., 1999; Krauss et al., 2005; Covault and Sanes, 1986). Three cell populations were obtained; muscle stem cells positive for α6ITG expression, muscle stem cells negative for

α6ITG expression and the non-myogenic cells expressing neither NCAM nor α6ITG (Fig. 2). Here, the α6ITG positive myogenic stem cells showed a robust myogenic differentiation capacity as indicated by the expression of MYOGENIN, DESMIN and MYHC. Interestingly, it was shown that expression of α6ITG expression is required for myogenic differentiation into myotubes. The α6ITG$^-$ cells showed cell elongation and alignment, but no myoblast fusion (Fig. 3). The myogenic commitment of the cell populations was further examined by determination of mRNA expression levels. *PAX7* expression was similar between the two myogenic NCAM$^+$ cell populations after isolation indicating that they both related to satellite cells. Interestingly, both α6ITG$^+$ and α6ITG$^-$ cells expressed the MRFs *MYF5* and *DESMIN*, indicating the myogenic commitment of both cell types. It has been shown that in murine muscle tissue, myogenic progenitor cells are subdivided in Pax7 expressing satellite cells lacking Myf5 and committed satellite cells co-



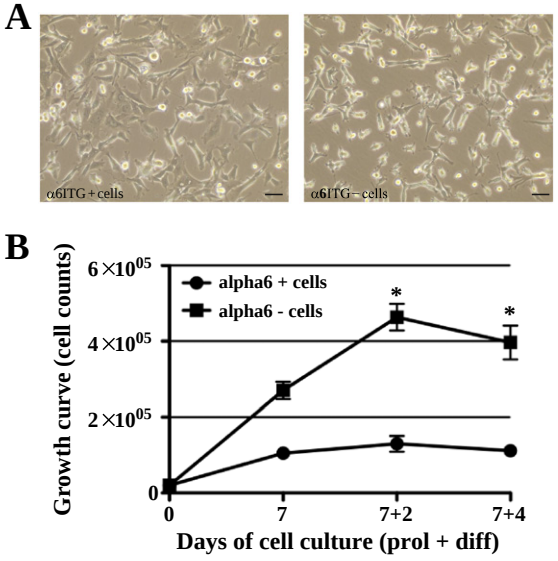


Figure 4 Cell culture. **A**. Both NCAM$^+$/ α6ITG$^+$ and NCAM$^+$/ α6ITG$^-$ populations showed a bi- and triangular shaped morphology with a more flattened appearance for α6ITG expressing cells. Scale bar is 50 μm. **B**. Growth curves represent cell amount during proliferating and differentiating. Cells that express α6ITG exhibit a lower proliferation rate (●) compared to cells without α6ITG expression (■. (*$p<0.0001$). Experimental outcome represents three biological replicates.

expressing Myf5 (Kuang et al., 2007). The hierarchy is based on the apical–basal position of the satellite cells towards the muscle fiber. Satellite cells identified as Pax7$^+$/ Myf5$^-$ with an orientation towards the basal lamina (basal-position) were demonstrated to express α7β1 integrins. Satellite cells with an apical orientated position towards the plasma membrane of the myofiber expressed Myf5 (Kuang et al., 2007). These Myf5$^+$ cells were indicated as a committed phenotype due to the loss of contact with the basal lamina and extracellular environment or niche, which is important in sustaining stem cell identity. Interestingly, in our experiments the α6ITG$^-$ cells expressed significantly higher levels of *A7ITG* and lower levels of *MYF5* compared to α6ITG$^+$ cells. This suggests that α6ITG$^-$ cells could have a basal orientated position and not yet directed into the myogenic program. This indicates that these cells are uncommitted early muscle precursor cells whereas the α6ITG$^+$ cells could be the myogenic initiated myogenic progenitor cells. The non-myogenic cells lacking NCAM expression showed significant higher levels of *A5ITG*, which is the receptor for fibronectin in fibroblasts (Huveneers et al., 2008).

Examination of the proliferation capacity of the two populations revealed that α6ITG$^-$ cells proliferated at a higher rate than α6ITG$^+$ cells. The α6ITG$^+$ cells stopped proliferation and formed myotubes by cell fusion, while α6ITG$^-$ cells continued cell proliferation until confluence was reached (Fig. 4). The high proliferation capacity of α6ITG$^-$ cells is consistent with the involvement of α6 integrin in the negative regulation of cell growth (Sastry et al., 1996). The differentiation capacity revealed a diminished myogenic commitment by cells lacking α6ITG expression compared to the α6ITG positive cells. Whether the α6ITG negative cells will become less proliferative and more myogenic when α6ITG is introduced into these cells for instance by over-expression, or whether the α6ITG negative cells have already entered different differentiation pathways is currently unknown.

Monoclonal antibodies blocked α6ITG expression on primary muscle stem cells and prohibited myoblast fusion without affecting proliferation or the expression of muscle-specific genes. This indicates a direct inhibition of cell fusion and differentiation. Downregulation of α6ITG mRNA expression using siRNA oligonucleotides confirmed the role α6ITG in myogenic differentiation.

Besides muscle satellite cells, several muscle progenitor cells have been identified so far among which are blood-vessel associated myo-endothelial cells (Zheng et al., 2007; Crisan et al., 2008), meso-angioblasts (Minasi et al., 2002) and muscle-derived stem cells (MDSCs) (Qu-Petersen et al., 2002). Interestingly, the MDSCs with the highest in vivo regeneration capacity are the most proliferative in vitro, similar to the α6ITG$^-$ population (Qu-Petersen et al., 2002). Early preplate (EP) cells, characterized as late myogenic precursors in vitro (Qu-Petersen et al., 2002), are comparable with the myogenic α6ITG+population. Differences in α6ITG expression could attribute to the in vitro and in vivo potentials and α6ITG can therefore be a useful target to

Figure 3 Characterization of sorted cell populations. Gene expression levels were determined by qRT-PCR on NCAM$^+$/ α6ITG$^+$, NCAM$^+$/ α6ITG$^-$ and NCAM$^-$/ α6ITG$^-$ cells relative to unsorted primary muscle cells. **A**. Myogenic differentiation capacity of α6ITG$^+$ cells after 4 days of induction. MYOGENIN (left, in green) detection on terminal differentiated muscle stem cells. DESMIN (middle, in green) detection represents formation of large multinucleated myotubes (arrow). Formation of MYHC (right, in green) expressing striated myotubes (arrow head). DNA is visible in blue, scale bar represents 20 μm. **B.** Immunofluorescent cell staining with anti-α6ITG antibodies (GoH3) on cytospins of the sorted cell populations (NCAM$^+$/ α6ITG$^+$, left upper panels; NCAM$^+$/ α6ITG$^-$, left lower panels) visualizes the expression of α6ITG in the α6ITG$^+$ cells (red). After 2 days of inducing differentiation α6ITG$^+$ cells formed myotubes (arrow; upper right panel), whereas cells lacking α6ITG remained aligned showing no cell fusion (lower right panel). Nuclei are visualized with DAPI staining (blue). Scale bar is 50 μm. **C.** The *A6ITG* was expressed at significantly higher levels in α6ITG$^+$ cells, *A5ITG* was expressed in NCAM$^-$/ α6ITG$^-$ (non-myogenic cells) at significantly higher levels, while *A7ITG* was significantly higher expressed in α6ITG$^-$ cells. Similar expression levels of *B1ITG* were detected between both α6ITG$^+$ and α6ITG$^-$ cell but the non-myogenic cells expressed significantly higher levels of *B1ITG*. Expression levels of *PAX7* were detected in α6ITG$^+$ and α6ITG$^-$ cells which were significantly higher compared to non-myogenic cells. A significantly higher *MYF5* expression level was detected in α6ITG$^+$ cells, while expression of *DESMIN* was significantly higher in α6ITG$^-$ cells. Experiment is performed in three biological replicates, in which the qRT-PCR analysis is run as technical triplicates. Asterisks denote significant differences in gene expression between the sorted population (NCAM$^+$/ α6ITG$^+$, NCAM$^+$/ α6ITG$^-$ and NCAM$^-$/ α6ITG$^-$) (ns = no significance, * $p<0.05$, * $p<0.01$, *** $p<0.001$).

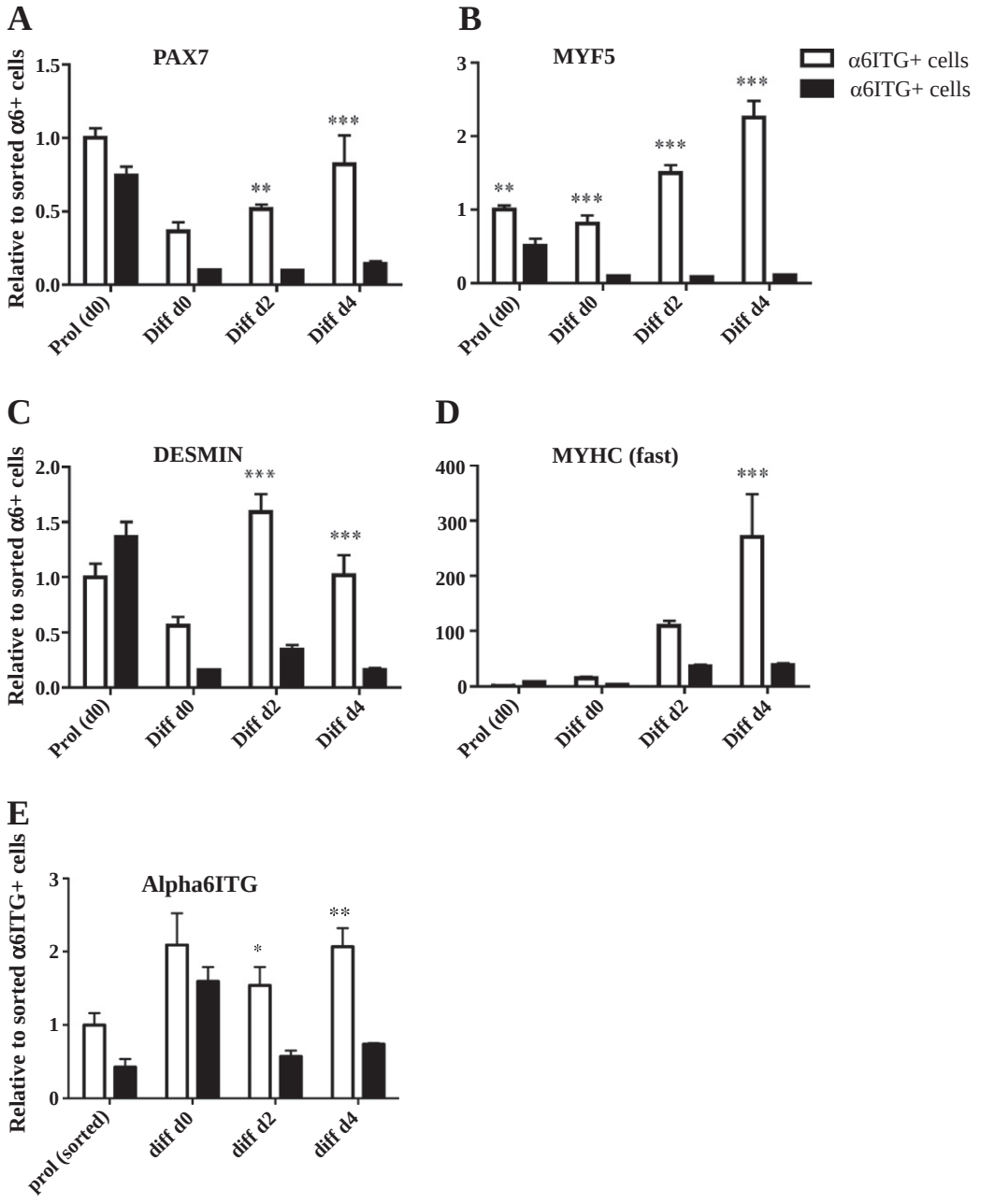


Fig. 5 Differentiation capacity of NCAM$^+$/ α6ITG$^+$ and NCAM$^+$/ α6ITG$^-$ cells. Expression levels during differentiation (diff) are relative to α6ITG$^+$ cells at day 0 (prol d0). **A**. *PAX7* expression. **B**. *MYF5* expression. **C**. *DESMIN* expression. **D**. *MYHC fast type* expression. E. *Alpha6ITG* expression. Experiment is performed in three biological replicates, in which the qRT-PCR analysis is run as technical triplicates. Asterisks denote significant differences in gene expression at the specific time point between the sorted population NCAM$^+$/ α6ITG$^+$ and NCAM$^+$/ α6ITG$^-$ (** $p<0.01$, *** $p<0.001$).

improve muscle regeneration. Studying similarities and differences between the α6ITG positive and α6ITG negative cell populations such as characterizing differentiation potential and in vivo exposure would attribute to a presumable role for α6ITGs on in vivo engraftment, survival and self-renewal. Discrimination between behavioral in vivo cell fate would indicate a preference to re-enter a quiescent satellite cell position, which could be determined by cell location towards the muscle fiber and *Sprouty* expression (Shea et al., 2010), or a more differentiated characteristic by fusion with myotubes showing a more myogenic regenerative potential.

Here, two types of stem cells have been identified in porcine muscle: those that express α6ITG and those that lack expression of this integrin type. Cells expressing α6ITG have an advantage in the formation of myotubes, possibly by an improved cell fusion capacity. Whether the cells that lack α6ITG will eventually start to express this integrin or whether

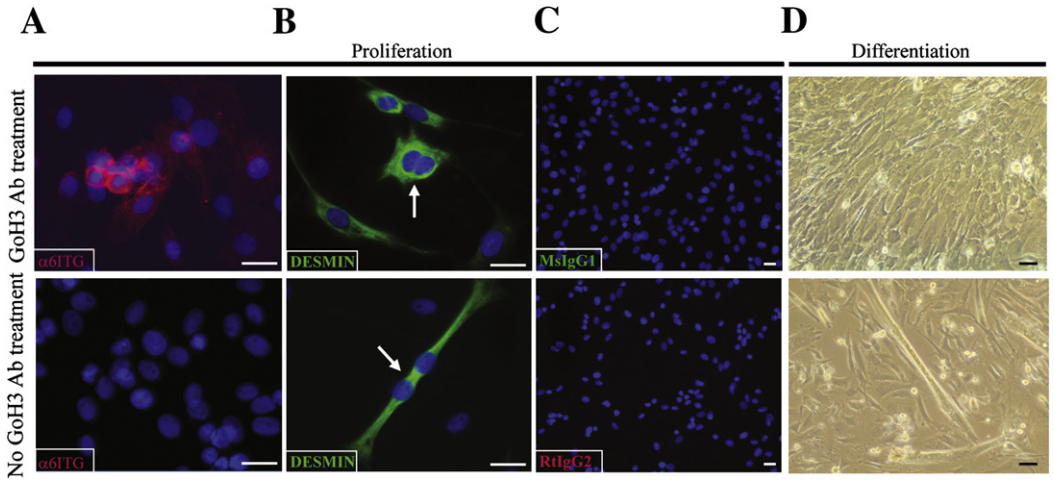


Fig. 6 Inhibition of α6ITG function using the rat-GoH3 antibodies. Upper row depicts primary muscle stem cells treated with GoH3 antibodies directed against α6ITG. Lower row depicts primary muscle cells without GoH3 antibody treatment. **A**. Detection of GoH3 antibodies on primary muscle stem cells using Alexa568-labeled secondary antibody against rat-IgGs. **B**. Visualization of DESMIN expression indicates adhesion of dividing myoblasts (arrows) on the cover slips. **C**. Isotype antibody incubations served as negative control for aspecific antibody binding. Nuclei were stained with DAPI (blue), scale bars represent 20 μm. **D**. No myotubes were observed in GoH3 treated primary muscle stem cells after 6 days of differentiation, while cells lacking GoH3 antibody treatment formed myotubes in culture. Scale bars represent 50 μm. Experimental outcome represents three biological replicates.

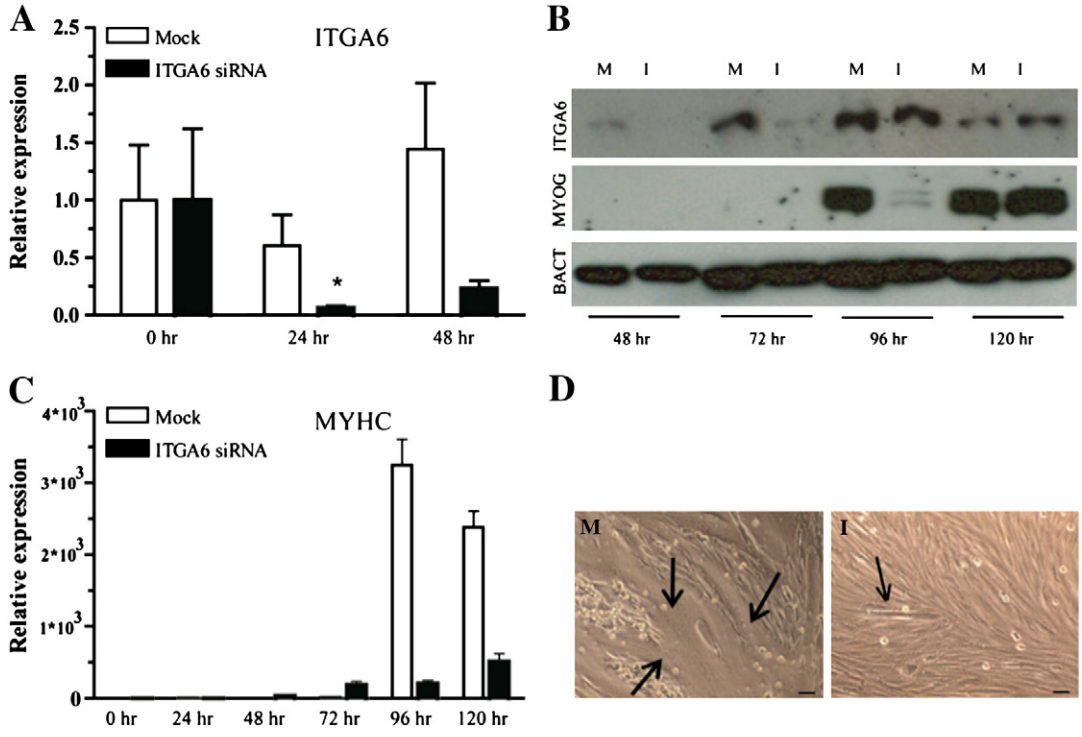


Fig. 7 Inhibitory effects of α6ITG downregulation on myogenic differentiation capacity of primary muscle stem cells. **A.** Messenger RNA expression levels of α6ITG relative to expression levels directly after transfection (0 h) determined by qRT-PCR. Efficient downregulation of α6ITG has been obtained by using ITGA6 siRNA. **B.** Protein levels determined by western blotting confirm silencing of α6ITG during 96 h post transfection. Myogenic determination was observed in the mock group by MYOGENIN (MYOG) expression. **C.** Quantitative RT-PCR confirmed myogenic differentiation of cells transfected with control siRNA by increased levels of MYHC expression in time. **D**. Morphological differentiation (myotube formation, arrows) was observed in the mock control group (M), which was very poor in cells with downregulated α6ITG expression (I). BACT = beta actin. MYHC = myosin heavy chain 2. Scale bars represent 50 μm. (* $p<0.01$). Experiment is performed in three biological replicates.

these cells have a different role in skeletal muscle is not known. The functions of integrins can be different in the various stem cell types predisposed by the extracellular environment and developmental progression. It has, for instance, been established that α6/β1 integrins are markers of neural stem cells, and that expression of these integrins decreases in differentiated neural cell types (Hall et al., 1990), which is opposite to our finding. However, skeletal muscle cells are a different type of cells that require fusion of progenitor cells during maturation. We propose that α6ITG is particularly important to establish fusion of the muscle progenitor cells important for differentiation of skeletal muscle.

In this study, it is proposed that α6ITG could serve as a biomarker to select for highly myogenic stem cells that may be used in experimental stem cell therapy. It should however be investigated whether cells expressing α6ITG lead to better engraftment than α6ITG negative cells, since successful engraftment encompasses more than in vitro differentiation efficiency.

4. Materials and methods

4.1. Isolation of porcine primary muscle stem cells

Porcine primary muscle stem cells were isolated from semitendinosus muscle of euthanized (intracardial injection of 0.15 ml/kg T61) piglets (hybrid York boars; 3 months of age). The dissected muscle parts were minced with scalpels after removal of adipose and connective tissues. Minced muscle tissue (50 g in total) was centrifuged (5 min, 2000 g) in phosphate-buffered saline (PBS, Braun, Melsungen, Germany) containing 1% HEPES and a cocktail of anti-bacterial agents (50 μg/ml gentamycin, 1% antibiotic-antimycotic mix and 250 ng/ml fungizone (all from Sigma ST Louis, MO), referred to as PBS^+-H. Muscle tissue parts were digested in 100 ml preheated 1 mg/ml protease solution (from *Streptomyces griseus,* Sigma, ST Louis, MO) in PBS^+-H for 60 minutes at 37 °C under repeated shaking as described previously (Doumit and Merkel, 1992; Gharaibeh et al., 2008). To further homogenize the tissue, trituration with 5 ml pipettes was performed. Undigested larger muscle parts were collected by modest centrifugation (5 min at 200 g) and stored on ice. Supernatant, containing single cells, was washed twice (10 min at 2000 g) in growth medium (GM) [Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG, Invitrogen, Carlsbad, CA), 20% fetal bovine serum (FBS, Invitrogen) and 50 μg/ml gentamycin]. The cells were filtered with cell strainers (pore size 70 μm, BD Falcon, Erembodegem, Belgium) and subsequently pelleted (10 min at 2000 g). Cell pellets were stored on ice. Further dissociation of cells from the remaining undigested muscle parts was performed by a 1 hour incubation with 0.15% w/v collagenase XI (Sigma) in DMEM-HG with 1% HEPES and 5% FBS. During incubation tissue solution was repeatedly triturated with 10 ml pipettes. Finally, the fully digested tissue was filtered (70 μm cell strainer), washed in GM and pelleted (10 min at 2000 g). To shock the erythrocytes both cell pellets were pooled in 20 ml cold hypotonic buffer (0.2 M NH_4Cl, 13 mM $KHCO_3$, pH 7.4) for 10 min on ice and centrifuged in cold PBS^+-H (5 min at 1000 g). Cells were taken up in GM, filtered through a 40 μm cell strainer (BD Falcon) to remove small tissue debris and subsequently collected by pelleting (5 min at 700 g). Cells were pre-plated in GM in addition of antibiotic–antimycotic mix and fungizone in uncoated T175 flasks (Corning Life Sciences, Amsterdam, The Netherlands) for the exclusion of fast-adhering fibroblasts during 1 h (37 °C, 5% CO_2). Cells that had not adhered were collected and stored in liquid nitrogen until further use (Qu et al., 1998). All steps were performed at room temperature (RT), unless otherwise mentioned.

4.2. Muscle stem cell proliferation and differentiation

For cell expansion primary muscle stem cells were cultured in 1 mg/ml Matrigel (Matrigel™ Basement Membrane Matrix; mouse tumor; phenol-red free, BD Bioscience, Bedford, MA) coated flasks (Sigma) in GM containing 5 ng/ml human basic fibroblast growth factor (bFGF; Sigma). Differentiation of the muscle stem cells was performed in differentiation medium [DM; DMEM-HG, 2% horse serum (HS, Invitrogen) with 50 μg/ml gentamycin] on laminin-coated (1 μg/ml) Lumox dishes (Ø 35 mm; Greiner Bio-One, Frickenhausen, Germany) or on laminin-coated 6-wells plates (Greiner Bio-One).

4.3. FACS sorting

Indirect cell staining with primary mouse antibodies against NCAM (5.1H11; 1:200 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA) and rat antibodies against α6ITG (GoH3; 2.5 μg/ml; BD) was used to select for specific cell populations by flow cytometry using a VantageTM SE flowcytometer (BD). Fluorescent conjugated secondary goat-anti mouse-PE and goat-anti rat-FITC antibodies were used to localize the primary antibodies. Isotype control antibodies (rat-IgG2 and mouse-IgG1; both 1:10 dilution; DakoCytomation, Glostrup, Denmark) were used to gate the fluorescence threshold for antibody specificity based on the visualization of nonspecific binding by the primary antibodies. Data were analyzed using FlowJo software (Oregon corporations, Ashland, OR).

4.4. Indirect immunofluorescence

After sorting by flow cytometry, cell populations were spun onto glass cover slides for 5 min at 800 rpm in 0.5% BSA in PBS using the Shandon Cytospin 4 (Thermo Scientific, Breda, The Netherlands). The cover slides were dried overnight and stored at −80 °C until further use. Furthermore, primary muscle stem cells were cultured for 6 days in GM on 1 $\mu g/cm^2$ laminin-coated (murine sarcoma; Sigma) glass cover slips and on laminin-coated Lumox dishes for 4 days in DM. Cells were fixed in cold methanol (2 min at −20 °C) for α6ITG staining. To stain DESMIN, MYOGENIN and Myosin heavy chain (MYHC) cells were fixed in 4% paraformaldehyde (PFA; Electron Microscopy Science, Hatfield, PA) for 15 min, followed by permeabilization in 0.5% Triton X-100 for 10 min. Snap-frozen muscle tissue was cryosectioned (5 μm), fixed in 4% PFA (15 min) and incubated for 30 min in blocking buffer [2% normal goat serum (DakoCytomation), 1% Bovine Serum Albumin (BSA; Roth, Karlsruhe, Germany), 0.1% fish gelatin (Sigma), 0.1% Triton X-100, 0.05% Tween-20 in PBS] to avoid aspecific antibody binding. Subsequently, cells were incubated with primary antibodies α6ITG (rat, clone GoH3; 10 μg/ml; BD), DESMIN (mouse, clone

D33; 10 μg/ml; DakoCytomation), MYOGENIN (mouse, clone F5D; 10 μg/ml; BD), MYHC (mouse, clone MY-32; 1:400; Sigma), PAX7 (mouse, clone PAX7; 10 μg/ml; R&D systems, MN) and Laminin (rabbit; 1:25; Sigma) in blocking buffer for 1 h. After washing in PBS with 0.05% Tween-20 (PBST; 3 times; 5 min) slides were incubated with Alexa488-labeled or Alexa568-labeled goat anti-mouse/ anti-rat/ anti-rabbit IgG (1:200; Invitrogen) for 45 min. Slides were washed in PBST, followed by counterstaining with DAPI (Invitrogen) to visualize the nuclei. Slides were dehydrated in an increasing alcohol series (70%, 90% and 100%; 3 min each), air-dried before being overlaid with FluorSave (Calbiochem, Darmstadt, Germany) and covered with glass. All antibody solutions were centrifuged for 10 min at 4 °C at full speed prior to use. All steps were performed at RT. Visualization of fluorescently labeled secondary antibodies was performed on a Leica DMRE fluorescence microscope.

4.5. Inhibition of α6ITG expression

On laminin expanded primary muscle stem cells were seeded at a density of 3000 cells/cm^2 into a laminin coated 12-wells plate. Blocking of α6ITG expression on primary muscle stem cells was performed in presence of the blocking antibody GoH3 (GoH3; 2.5 μg/ml; BD). After 8 days of cell culturing in GM differentiation was induced during 6 days in DM. Proliferation and differentiation medium was refreshed every third day with addition of GoH3 antibody.

Expression of α6ITG in primary muscle stem cells was downregulated by RNAi mediated knockdown. Cells were transfected with α6ITG siRNA duplex oligoribonuleotides (5′-GAAACGUGCUGUUCCAUAA-3′) using electroporation (1 time at 160 V, 70 ms pulse length; ECM 830 Electroporation System, Harvard Apparatus, Kent, UK). Universal control siRNA duplex oligoribonuleotides were used as a nonspecific control (mock) (all purchased from Eurogentec, Maastricht, The Netherlands). Cells were transfected with 500 pmol siRNA per 1×10^5 cells and seeded onto Matrigel-coated plates in a density of 5000 cells/cm^2 in GM. After 48 h myogenic differentiation was induced by medium replacement for DM. Cells were collected for RNA and protein isolation at 0 h to 120 h of culturing.

4.6. Quantification of gene expression

RNA isolation using a RNeasy Mini Kit (Qiagen, Valencia, CA) was either performed from directly sorted cell populations or from cell cultures which were first trypsinized (0.25% Trypsin-EDTA, Invitrogen), pelleted by centrifugation (5 min at 300 g) and stored at −80 °C before RNA isolation. Additionally, a second DNase treatment was performed on RNA samples by incubation with 2 μl DNase (Qiagen) for 25 min at 37 °C, followed by inactivation for 10 min at 70 °C. The cDNA was generated using Superscript™ III First-strand Synthesis System (Invitrogen) and quantitative real-time RT-PCR (qRT-PCR) performed on cDNA (1 μl) using an iCycler with iQ™ SYBR® Green supermix (both from BIO-RAD, Hercules, CA). A 12.5 pmol primer concentration per 25 μl reaction amplified cDNA after a denaturation step of 3 min at 95 °C in a 40 cycle protocol [30 s at 95 °C, 20 s 51–63 °C (Table 1 Wilschut et al., 2010; Wilschut et al., 2008; Kuijk et al., 2007), 30 s at 72 °C and subsequently 77 repeats of 15 s with a 0.5 °C increase in temperature after every repeat starting at 60 °C]. Primer specificity was confirmed by product sequencing. For relative gene expression quantification samples were normalized against mRNA levels of reference genes (*GAPDH*, *UBQ* and *PGK1*) using GeNorm software (Primer Design

Table 1 Overview of primers used for quantitative RT-PCR.

Gene	Primer sequence 5′→3′	Amplicon size	Ta (°C)	GenBank accession no.
ITGA5	F: GCCTGCAAAGATCTGTCCTC R: AGAGTTCCCCTTGGAGTGGT	215 bp	58	XM_001925252 (Wilschut et al., 2010)
ITGA6	F: AAACGAGAAATTGCTGAAAGAC R: CACTAGAATGATCCACCAAGG	365 bp	54	NM_001109981 (Wilschut et al., 2010)
ITGA7	F: CAGAGCTGGCTGCTGGTG R: CTGGTCGATGTCCACTCTGT	132 bp	60	XM_001916598 (Wilschut et al., 2010)
ITGB1	F: ATGAGGAGGATTACTTCAGACTTC R: GCAGCCGTGTCACATTCC	304 bp	53	NM_213968
PAX7	F: GGTGGGGTTTTCATCAATGG R: GTCTCTTGGTAGCGGCAGAG	155 bp	55	AY653213 (Wilschut et al., 2008)
MYF5	F: TGCCATCCGCTACATTGAGAG R: GTTCTTTCGGGACCAGACAGG	160 bp	55	Y17154 (Wilschut et al., 2010)
DESMIN	F: CCGAGATCTACGAGGAGGAG R: TCTCGGCTTCTTCTTTCAGC	162 bp	55	AF363284 (Wilschut et al., 2010)
MYHC-2	F: CCGTCTGGATGAGGCTGAG R: CCGTCTGGTAGGTGAGTTCC	179 bp	60	NM_214136 (Wilschut et al., 2008)
PGK1	F: AGATAACGAACAACCAGAGG R: TGTCAGGCATAGGGATACC	126 bp	56.4	AY677198 (Kuijk et al., 2007)
UBQ	F: TTCGTGAAGACCTTGACTG R: GGACTCCTTCTGGATGTTG	186 bp	51	M18159 (Kuijk et al., 2007)
GAPDH	F: TCGGAGTGAACGGATTTG R: CCTGGAAGATGGTGATGG	219 bp	51	AF017079 (Kuijk et al., 2007)

Ltd., Southampton, UK) (Vandesompele et al., 2002). Gene expression levels of siRNA-transfected cells and mock-transfected cells were normalized against PGK1 expression levels and plotted relatively to expression levels determined directly after transfection (0 h) (Fig. 7). Results were analyzed using GraphPad prism in a two-way ANOVA with a Bonferroni post-test.

4.7. Immunoblotting

Cells were washed with cold PBS and lysed in buffer containing 140 mM NaCl, 50 mM Tris–HCL pH7.5, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol and protease inhibitor cocktail 1 tablet/50 ml lysis buffer (Roche Applied Sciences, Almere, The Netherlands). Protein measurement was performed with a DC protein assay (Bio-Rad, Veenendaal, The Netherlands). Equal amounts (3.5 μg) of reduced (5 min at 95 °C) protein lysate was separated onto 12% Tris–HCl PAGE gels and blotted to Trans-Blot nitrocellulose transfer membranes (Bio-Rad). Membranes were blocked in 5% Blotting Grade Blocker non-fat dry milk in 1x PBS-0.1% Tween X-100 for 1 h and incubated with primary antibodies against ITGA6 (rabbit polyclonal, 1:1000; Cell Signaling Technology, Danvers, MA), MYOGENIN (F5D, mouse, 2 μg/ml, BD) and BACT (rabbit polyclonal; 1:1000; Cell Signaling Technology). Secondary incubation for 1 h with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) was followed by SuperSignal West Dura Extended Duration Substrate (Pierce, ThermoScientific, Erembodegem, Belgium) after which the blots were exposed to autoradiography film (Kodak, Rochester, NY).

Author disclosure statement

No potential conflicts of interest.

Acknowledgments

This work was supported by a SenterNovem grant from the Ministery of Economic Affairs. The NCAM antibody (5.1H11) developed by H.M. Blau and F.S. Walsh was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. KW was responsible for the design, experimental procedures and was the primary author for the manuscript. HT largely contributed to the RNAi silencing procedures and GA supported the flow cytometric procedures. HH supervised the design and analysis. BR supervised the design, analysis and writing.

References

Alapat, D.V., et al., 2009. Fiber-types of sarcomeric proteins expressed in cultured myogenic cells are modulated by the dose of myogenin activity. Cell. Signal. 21 (1), 128–135.

Aumailley, M., Timpl, R., Sonnenberg, A., 1990. Antibody to integrin alpha 6 subunit specifically inhibits cell-binding to laminin fragment 8. Exp. Cell Res. 188 (1), 55–60.

Bajanca, F., et al., 2004. Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Dev. Dyn. 231 (2), 402–415.

Bajanca, F., et al., 2006. Integrin alpha6beta1-laminin interactions regulate early myotome formation in the mouse embryo. Development 133 (9), 1635–1644.

Bao, Z.Z., et al., 1993. Alpha 7 beta 1 integrin is a component of the myotendinous junction on skeletal muscle. J. Cell Sci. 106 (Pt 2), 579–589.

Blanton Jr., J.R., et al., 1999. Isolation of two populations of myoblasts from porcine skeletal muscle. Muscle Nerve 22 (1), 43–50.

Buckingham, M., et al., 2003. The formation of skeletal muscle: from somite to limb. J. Anat. 202 (1), 59–68.

Capkovic, K.L., et al., 2008. Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation. Exp. Cell Res. 314 (7), 1553–1565.

Cerletti, M., et al., 2008. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell 134 (1), 37–47.

Charlton, C.A., Mohler, W.A., Blau, H.M., 2000. Neural cell adhesion molecule (NCAM) and myoblast fusion. Dev. Biol. 221 (1), 112–119.

Covault, J., Sanes, J.R., 1986. Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. J. Cell Biol. 102 (3), 716–730.

Crisan, M., et al., 2008. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. Methods Cell Biol. 86, 295–309.

Danen, E.H., Sonnenberg, A., 2003. Integrins in regulation of tissue development and function. J. Pathol. 201 (4), 632–641.

Doumit, M.E., Merkel, R.A., 1992. Conditions for isolation and culture of porcine myogenic satellite cells. Tissue Cell 24 (2), 253–262.

Gharaibeh, B., et al., 2008. Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. Nat. Protoc. 3 (9), 1501–1509.

Gullberg, D., et al., 1998. Integrins during muscle development and in muscular dystrophies. Front. Biosci. 3, D1039–D1050.

Hall, D.E., et al., 1990. The alpha 1/beta 1 and alpha 6/beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin. J. Cell Biol. 110 (6), 2175–2184.

Hayashi, Y.K., et al., 1998. Mutations in the integrin alpha7 gene cause congenital myopathy. Nat. Genet. 19 (1), 94–97.

Hemler, M.E., 1999. Dystroglycan versatility. Cell 97 (5), 543–546.

Hierck, B.P., et al., 1993. Variants of the alpha 6 beta 1 laminin receptor in early murine development: distribution, molecular cloning and chromosomal localization of the mouse integrin alpha 6 subunit. Cell Adhes. Commun. 1 (1), 33–53.

Huveneers, S., et al., 2008. Binding of soluble fibronectin to integrin alpha5 beta1 – link to focal adhesion redistribution and contractile shape. J. Cell Sci. 121 (Pt 15), 2452–2462.

Hynes, R.O., 2002. Integrins: bidirectional, allosteric signaling machines. Cell 110 (6), 673–687.

Krauss, R.S., et al., 2005. Close encounters: regulation of vertebrate skeletal myogenesis by cell–cell contact. J. Cell Sci. 118 (Pt 11), 2355–2362.

Kuang, S., et al., 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129 (5), 999–1010.

Kuijk, E.W., et al., 2007. Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. BMC Dev. Biol. 7, 58.

Le Bellego, F., et al., 2002. Laminin-alpha6beta1 integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells. J. Endocrinol. 172 (1), 45–59.

McKinnell, I.W., et al., 2008. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat. Cell Biol. 10 (1), 77–84.

Menko, A.S., Boettiger, D., 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell 51 (1), 51–57.

Mesires, N.T., Doumit, M.E., 2002. Satellite cell proliferation and differentiation during postnatal growth of porcine skeletal muscle. Am. J. Physiol. Cell Physiol. 282 (4), C899–C906.

Minasi, M.G., et al., 2002. The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development 129 (11), 2773–2783.

Montarras, D., et al., 2005. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309 (5743), 2064–2067.

Oustanina, S., Hause, G., Braun, T., 2004. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J. 23 (16), 3430–3439.

Pownall, M.E., Gustafsson, M.K., Emerson Jr., C.P., 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu. Rev. Cell Dev. Biol. 18, 747–783.

Qu, Z., et al., 1998. Development of approaches to improve cell survival in myoblast transfer therapy. J. Cell Biol. 142 (5), 1257–1267.

Qu-Petersen, Z., et al., 2002. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J. Cell Biol. 157 (5), 851–864.

Relaix, F., et al., 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435 (7044), 948–953.

Rudnicki, M.A., et al., 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75 (7), 1351–1359.

Sastry, S.K., et al., 1996. Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. J. Cell Biol. 133 (1), 169–184.

Seale, P., et al., 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102 (6), 777–786.

Shea, K.L., et al., 2010. Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. Cell Stem Cell 6 (2), 117–129.

Sherwood, R.I., et al., 2004. Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 119 (4), 543–554.

Sonnenberg, A., Modderman, P.W., Hogervorst, F., 1988. Laminin receptor on platelets is the integrin VLA-6. Nature 336 (6198), 487–489.

Sonnenberg, A., et al., 1990. Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that alpha 6 beta 1 but not alpha 6 beta 4 functions as a major receptor for fragment E8. J. Cell Biol. 110 (6), 2145–2155.

Tajbakhsh, S., 2005. Skeletal muscle stem and progenitor cells: reconciling genetics and lineage. Exp. Cell Res. 306 (2), 364–372.

Tamaki, T., et al., 2002. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J. Cell Biol. 157 (4), 571–577.

van der Flier, A., Sonnenberg, A., 2001. Function and interactions of integrins. Cell Tissue Res. 305 (3), 285–298.

Vandesompele, J., et al., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (7) p. RESEARCH0034.

von der Mark, H., et al., 1991. Skeletal myoblasts utilize a novel beta 1-series integrin and not alpha 6 beta 1 for binding to the E8 and T8 fragments of laminin. J. Biol. Chem. 266 (35), 23593–23601.

Webster, C., et al., 1988. Isolation of human myoblasts with the fluorescence-activated cell sorter. Exp. Cell Res. 174 (1), 252–265.

Wilschut, K.J., et al., 2008. Isolation and characterization of porcine adult muscle-derived progenitor cells. J. Cell. Biochem. 105 (5), 1228–1239.

Wilschut, K.J., Haagsman, H.P., Roelen, B.A., 2010. Extracellular matrix components direct porcine muscle stem cell behavior. Exp. Cell Res. 316 (3), 341–352.

Zammit, P.S., et al., 2006. Pax7 and myogenic progression in skeletal muscle satellite cells. J. Cell Sci. 119 (Pt 9), 1824–1832.

Zheng, B., et al., 2007. Prospective identification of myogenic endothelial cells in human skeletal muscle. Nat. Biotechnol. 25 (9), 1025–1034.