Sulf1A and HGF regulate satellite-cell growth

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Summary

The role of Sulf1A, sulfation and hepatocyte growth factor (HGF) in satellite-cell growth was examined in an in vitro model of dissociated whole skeletal muscle fibres. Pax7-positive quiescent satellite cells express little or no Sulf1A but show rapid re-expression in regenerating myoblasts and myotubes, similar to embryonic muscle and in vitro satellite cells preceding asynchronous MyoD activation. Once activated, Sulf1A and MyoD re-expression persists up to 72 hours in most satellite cells under normal culture conditions and following moderate changes in sulfation, whereas Sulf1A neutralisation by antibodies not only enhances satellite-cell proliferation but also downregulates MyoD and Pax7 expression in a large proportion of the satellite cells. The HGF exposure also induces similar but even more pronounced changes characterised by variable sulfation levels and rapid downregulation of MyoD and Pax7 without myogenin activation in a sub-set of cells. This Pax7-MyoD-myogenin-negative sub-population expresses Sulf1A and Myf5. The transfer of all such satellite-cell progenies onto gelatin-coated-substratum re-activates MyoD and Pax7 gene expression in all cells, thus detecting a distinct sub-population of satellite cells. We conclude that HGF and fine-tuned sulfation levels are major contributory factors controlling satellite-cell growth by regulating the relative activities of actively proliferating and differentiating cells.

Key words: Satellite cells, Muscle regeneration, Sulf1A, Sulfation, Hepatocyte growth factor

Introduction

Adult skeletal muscle fibres retain the ability to repair and regenerate injured muscle by activating a specialised class of resident muscle stem cells called satellite cells that are located under the basal lamina of each muscle fibre (Mauro, 1961). Such satellite cells, which are generated and put aside during foetal growth, are used for muscle repair and recruitment for postnatal muscle growth (Bischoff, 1986; Collins et al., 2005). The presence or recruitment of some other non-satellite stem cells has also been demonstrated in skeletal muscle, but the satellite-cell population remains the major source of cells contributing to muscle regeneration and maintenance (Collins et al., 2005; Otto et al., 2008). Although considerable information is available about cellular and molecular characteristics of both the quiescent and activated satellite cells, the contributions of different molecular signalling pathways that regulate satellite-cell proliferation and differentiation, however, remain poorly defined, and consequently their specific impact on the level of muscle regeneration still remains unclear. The level of muscle regeneration following injury would not only be dependent on the number of satellite cells but also the composition of surrounding extracellular matrix (ECM) harbouring specific growth factors and signalling molecules. The activities of such growth factors would be further regulated by the availability of appropriate primary and secondary receptors, including heparan sulfate proteoglycans (HSPGs), since many growth factors in addition to their specific primary receptors require HSPGs as co-receptors (Casar et al., 2004). HSPGs interact with growth factors and primary receptors through their heparan sulfate (HS) chains, the sulfation levels of which can regulate the activities of growth factors requiring HSPG interaction. HSPGs contain repeating disaccharide units of uronic acid linked to glucosamine (Bernfield et al., 1999; Prydz and Dalen, 2000) that are selectively sulfated at the N, 3-O and 6-O positions of glucosamine and the 2-O position of uronic acid residues by actions of sulfotransferases in the Golgi complex. The sulfation state of HS chains of HSPGs influences their interactions with signalling molecules. For example, fibroblast growth factor (FGF) signal transduction is dependent on the sulfation of 2-O and 6-O positions on HS chains. The 2-O sulfation is required for bFGF binding to heparin and 6-O sulfation for bFGF-dependent dimerisation and activation of the FGFR1 receptor, as revealed by both biochemical (Jemth et al., 2002) and crystal structure studies (Schlessinger et al., 2000) of FGF-FGFR1-heparin ternary complexes.

HSPG sulfation is also extracellularly regulated by Sulf1, a member of the sulfatase gene family, related to the lysosomal *N*-acetyl glucosamine sulfatases (Lukatela et al., 1998; Knaust et al., 1998; Robertson et al., 1992) that has been shown to modulate the activities of many growth factors and signalling molecules by regulating the sulfation status of specific HSPGs as it specifically reduces the 6-O-sulfation of HS, a component of the HSPGs. For example, FGF activity has been shown to be inhibited by Sulf1 during chick angiogenesis and in ovarian cells by removing 6-O sulfates from glucosamine residues in HS of HSPGs, required as secondary receptors for FGF2 and FGF4 function (Morimoto-Tomita et al., 2002; Ai et al., 2003; Lai et al., 2004; Wang et al., 2004). Similarly, Sulf1 has been shown to attenuate the activation of both ERK-MAP kinases and AKT signalling mediated by HGF (Lai et al., 2004) because HGF is also a heparin-binding growth factor regulated by sulfation of cell-surface HSPGs.

Both HGF and FGFs are known to regulate satellite-cell activation and proliferation (Kästner et al., 2000; Sheehan et al., 2000; Cornelison et al., 2001; Tatsumi and Allen, 2004) and since their activities are modulated by Sulf1, we decided to investigate the role of Sulf1 and sulfation in satellite-cell activation and proliferation using cultured muscle fibres as a model of

regeneration. In a recent study we described a new shorter Sulf1 splice variant that we named Sulf1B to distinguish it from the previously described full-length Sulf1 that we re-named Sulf1A (Sahota and Dhoot, 2009). Although reverse transcriptase (RT)-PCR analysis demonstrated low-level Sulf1B expression in skeletal muscle cells, immunocytochemical investigation only detected Sulf1A expression in myogenic cells (Sahota and Dhoot, 2009). The immunocytochemical staining in the present study also showed the presence of only Sulf1A in satellite-cell-derived myogenic cells since no differences were observed when the same tissue sections or satellite cells were also stained with another antibody that reacts with both Sulf1A and Sulf1B isoforms (Sahota and Dhoot, 2009). Therefore, we specifically examined the expression and role of Sulf1A in this study. Sulf1A is barely detectable in adult skeletal muscle fibres but is expressed in embryonic myogenic cells from which the Sulf1A gene was first isolated during an earlier investigation (Dhoot et al., 2001). Our earlier study also demonstrated the relationship of MyoD activation to Sulf1A expression, since the inhibition of Sulf1 by antisense oligonucleotides markedly downregulated MyoD expression in early developing somites (Dhoot et al., 2001). MyoD is a basic helix-loop-helix transcription factor, which is one of the four myogenic regulatory growth factors required for myogenesis, that can be used as a marker of myogenic activation.

To examine the role of Sulf1A, sulfation and HGF in satellite-cell growth, satellite cells on dissociated whole skeletal muscle fibres were grown in normal culture medium and in the presence of chemically and immunochemically induced changes in sulfation levels and HGF exposure. The quiescent satellite cells in this study were identified by the expression of Pax7, a widely used marker, believed to be ubiquitously expressed in this cell population. Pax7 is required for the maintenance of postnatal skeletal muscle since the satellite cells are depleted in the adult *Pax7*$^{-/-}$ mouse (Seale et al., 2004; Zammit et al., 2006). The present study demonstrates Sulf1A re-expression in regenerating muscle and rapid Sulf1A activation in vitro that precedes asynchronous MyoD activation. Not only exposure to HGF but also a reduction in Sulf1A levels by neutralising antibodies induces greatly enhanced satellite-cell proliferation and a sub-population of satellite-cell progeny characterised by rapid Pax7 and MyoD downregulation without myogenin activation.

Results

Sulf1A is undetectable in quiescent satellite cells but is re-activated in regenerating myoblasts and myotubes recapitulating early muscle development

Sulf1A is generally undetectable in normal adult skeletal muscles using immunocytochemical procedure (Fig. 1A,B and Fig. 2) but is expressed at high levels in embryonic myogenic cells (Fig. 1C) during skeletal muscle development (Dhoot et al., 2001; Zhao et al., 2006; Sahota and Dhoot, 2009). The non-staining of mature muscle fibres and their satellite cells with our three different Sulf1 antibodies (Sahota and Dhoot, 2009) was clearly not due to their poor avidity as these antibodies stained blood capillaries quite dark in the same tissue sections (Fig. 1A,B). For example, although Sulf1A protein was undetectable in satellite cells in anterior latissimus dorsi (ALD) muscle of a 3-week-old chicken (Fig. 1A) and adult mouse extensor digitorum longus (EDL) muscle fibres (Fig. 1B), Sulf1A expression in the same tissues was clearly apparent in endothelial cells of blood capillaries. The presence of satellite cells in both muscle tissue sections and isolated single muscle fibres that did not stain for Sulf1A was confirmed from their positive staining for Pax7, a known ubiquitously expressed marker of satellite cells (Seale et al., 2004; Zammit et al., 2004). Although Sulf1A expression was not detected in either the quiescent satellite cells or mature muscle fibres in adult muscle, its expression at both mRNA and protein levels was easily apparent in experimentally injured post-hatch chicken (Fig. 1D) as well as spontaneously regenerating myogenic cells in postnatal mdx (dystrophin-deficient) mouse muscles when investigated using in situ hybridisation or immunocytochemical procedures (Fig. 1E). For example, larger original mature muscle fibres are unstained for Sulf1A mRNA (black asterisk), smaller regenerating myotubes are stained (blue colour, white asterisk), for Sulf1A mRNA (Fig. 1Ei,ii) with the level of Sulf1A expression varying in individual regenerating myotubes. Sulf1A restriction to only the regenerating myotubes was not only apparent by in situ hybridisation but also immunocytochemically using Sulf1 antibodies (Sahota and Dhoot, 2009) to locate Sulf1A protein expression (Fig. 1Eiii,iv), whereas undamaged larger original muscle fibres did not show any Sulf1A expression.

Sulf1A is undetectable in Pax7-positive quiescent satellite cells but is rapidly re-activated in vitro preceding non-synchronous MyoD activation

The relationship of Sulf1A to Pax7 and MyoD expression was further investigated in vitro to examine whether changes in growth factors or sulfation levels induce changes in Sulf1A or either of these two transcription factors. Although little or no Sulf1A expression was observed in satellite cells on freshly isolated single fibres, Sulf1A expression in satellite cells was readily observed between 9 and 72 hours with the level of Sulf1A reducing in a small sub-set of the satellite cells (<2%) by 72 hours (Fig. 2). Sulf1A expression in satellite cells first became apparent at 8-9 hours in vitro but we did not observe MyoD expression at this stage using mouse monoclonal antibody 5.8A with our staining protocol. MyoD activation, however, became easily apparent at 12 hours using this antibody. The difference in MyoD activation in satellite cells relative to Sulf1A expression was not only apparent between 9 and 12 hours but also in selective sub-sets of satellite cells during later periods of in vitro growth. For example, a proportion of Sulf1A-positive (Sulf1A^{+}) satellite cells were still MyoD-negative (MyoD^{-}) at 24 or 30 hours in vitro (cells indicated by arrows in Fig. 2). Between 9 and 30 hours of in vitro growth, at least 20% of the satellite cells identified on the basis of Sulf1A staining were still MyoD^{-}, further confirming the later and asynchronous onset of MyoD activation. At 48 hours, virtually all satellite cells stained equally darkly for both Sulf1A and MyoD (Fig. 2). A large majority of the satellite cells also showed dark staining for both Sulf1A and MyoD at 72 hours under normal control culture conditions although some MyoD^{-} cells (~3%) were observed at this stage. MyoD expression, however, still appeared fairly high in most of the satellite cells at 72 hours in vitro.

Unlike Sulf1A, high Pax7 expression was detected not only in freshly isolated single fibres but also in all satellite cells at all stages until 48 hours of culture using normal growth medium. At 72 hours, a large majority of the satellite cells still stained for Pax7 although the level of Pax7 expression varied considerably in different satellite cells (Fig. 2), with some cells showing quite low-level expression at this stage. Nevertheless, a large majority of the satellite-cell-derived progeny (over 97%) still expressed Pax7

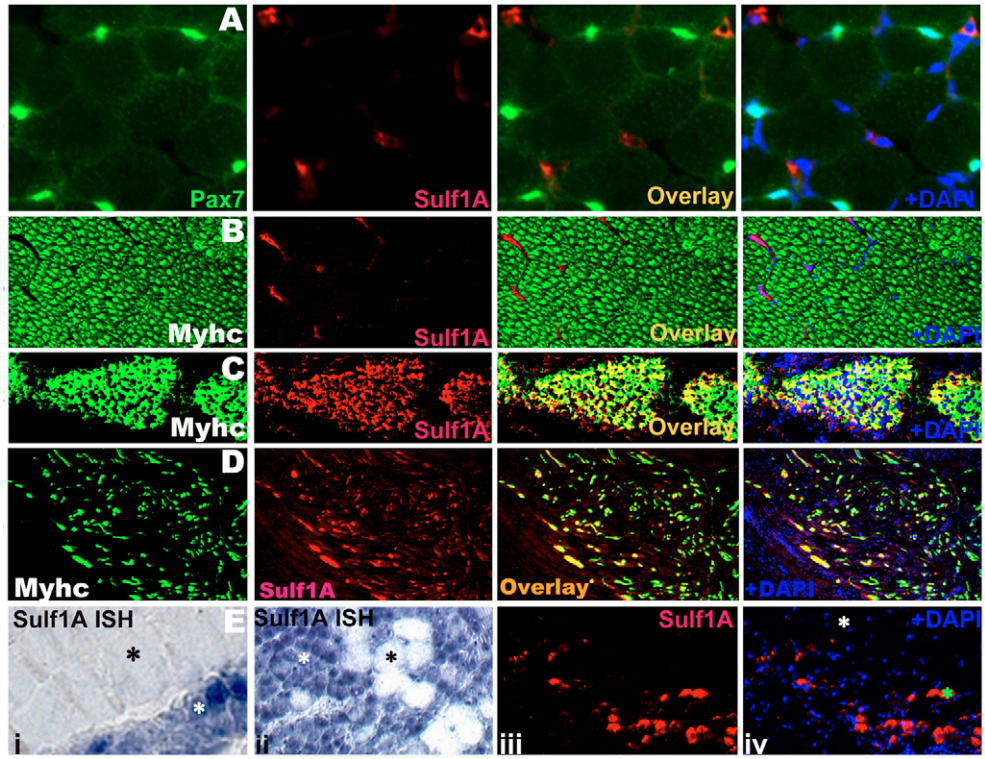

Fig. 1. Sulf1A is undetectable in quiescent satellite cells and adult muscle fibres but is re-activated in regenerating myotubes. Double immunofluorescence stained for Pax7 (green) or striated muscle type myosin heavy chain (green) and Sulf1A (red) of frozen sections of (**A**) an ALD muscle from a 3-week old chicken, (**B**) adult mouse EDL, (**C**) 13-day foetal mouse myotome, (**D**) 7-day post crush-injury chicken gastrocnemius muscle. and (**Eiii**,**iv**) paraffin sections of 27-day mdx mouse muscle. (Ei,ii) In situ hybridisation of mdx mouse muscle. The superimposed images in A (overlay) show that endothelial cells of blood capillaries stain positive for Sulf1A, but none of the Pax7-positive satellite cells show Sulf1A expression. Adult mouse muscle fibres (B), unlike foetal mouse myotubes (C), show no Sulf1A expression although Sulf1A expression is detectable in blood capillaries. Sulf1A expression in regenerating myotubes of experimentally injured chicken and mouse dystrophic muscle is apparent at the mRNA level (Ei,ii), using in situ hybridisation (ISH), as well as at the protein level (D and Eiii,iv) using immunofluorescence. The black asterisk in Ei,ii indicates a lack of staining in undamaged mature muscle fibres, and the white asterisk in the same sections indicates Sulf1A expression in regenerating myotubes as apparent from their central nuclei. The white asterisk in Eiv indicates an unstained original muscle fibre, and the green asterisk indicates Sulf1A staining of regenerating myotubes. For myosin heavy chain expression, antibody 83B6 was used in B and C, whereas antibody MF20 was used in D. Myhc, myosin heavy chain.

although the level of Pax7 was considerably reduced in some satellite cells by 72 hours in vitro.

Chemical changes in sulfation levels induce a moderate change in satellite-cell proliferation or MyoD- and Pax7-expression pattern

Sulf1A expression in activated satellite cells could relate to the HSPG sulfation levels required at different stages of development. We therefore exposed satellite cells on isolated single fibres in vitro to increased or decreased chemical sulfation by adding to the culture medium either 8 mM sodium sulfate (Fig. 3A-F), to increase sulfation, or 8 mM sodium chlorate (Fig. 3G-I), to chemically reduce HSPG sulfation. The satellite cells on single fibres grew well in the presence of 8 mM sodium sulfate, showing a slight increase in satellite-cell number per cluster when compared with the control (Fig. 3A-F and Fig. 7). Sulf1A expression, however, persisted in most satellite cells throughout the 72-hour culture period. Sulf1A levels in a large majority of the satellite cells appeared particularly high at 48 and 72 hours of culture (Fig. 3). Pax7 expression also persisted throughout the culture period of 72 hours although the levels of Pax7 expression varied during later stages (Fig. 3A-C). As was the case with Sulf1A and Pax7, MyoD expression also persisted in a large proportion of the satellite cells although MyoD was undetectable or present at reduced levels in a small proportion of the satellite-cell progeny (Figs 3, 7).

Unlike specific removal of 6-O sulfates by Sulf1A, chlorate de-sulfates HS at all positions. The satellite cells on isolated single fibres nevertheless were activated and grew reasonably well in the presence of 8 mM chlorate that greatly reduces HSPG sulfation. The number of satellite cells per cluster, however, was slightly reduced in the presence of 8 mM chlorate when compared with the control (Figs 3 and 7). Despite the global chemical reduction of sulfation by chlorate, some Sulf1A expression persisted in most satellite cells throughout the culture period although the level of Sulf1A, as apparent from immunocytochemical staining intensity, appeared reduced in all satellite cells at 72 hours. Pax7 (not shown) and MyoD expression (Fig. 3G-I) persisted in virtually all chlorate-treated satellite cells throughout the 72-hour culture period. The 8 mM chlorate concentration used in this study, resulted in greatly reduced sulfation of satellite cells (Fig. 3J) without inducing apoptosis or toxicity (not shown) compared with the routinely used levels of 10-30 mM in other studies (Cornelison et al., 2001) that also demonstrate no toxicity at such concentrations. The addition of 100

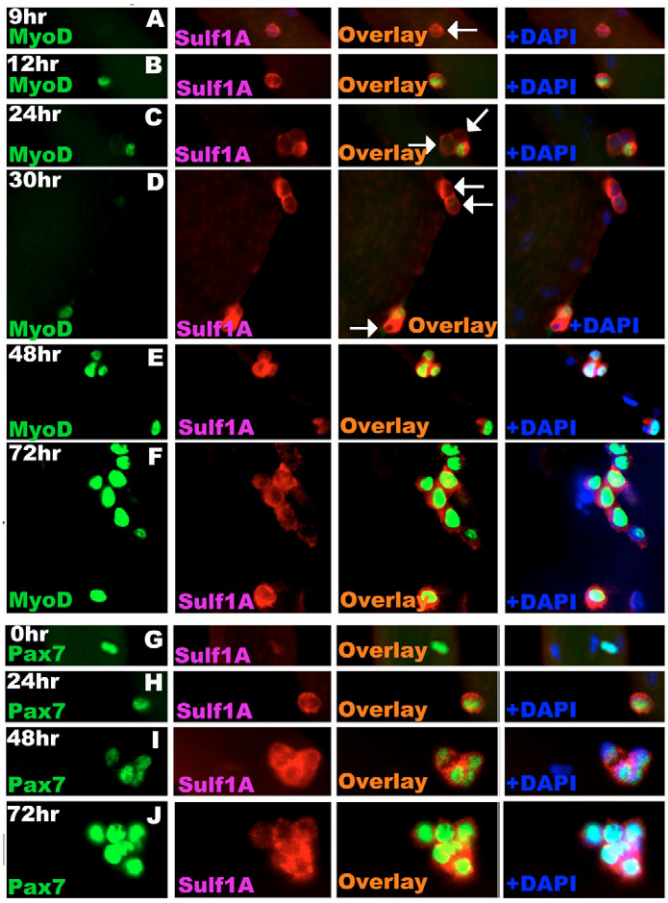

Fig. 2. Sulf1A activation in satellite cells precedes asynchronous MyoD activation in vitro. Satellite cells on dissociated single fibres stained after different time intervals in vitro for MyoD (column 1, **A-F**) or Pax7 (column 1 **G-J**) and Sulf1A (column 2) using a double immunofluorescence procedure. The superimposed images (overlay) of MyoD and Sulf1A as well as Pax7 and Sulf1A staining are shown in column 3 with the addition of DAPI staining in column 4. Some satellite cells, indicated by arrows at the 9-, 24- and 30-hour time points show Sulf1A staining but still no MyoD staining. hr, hours.

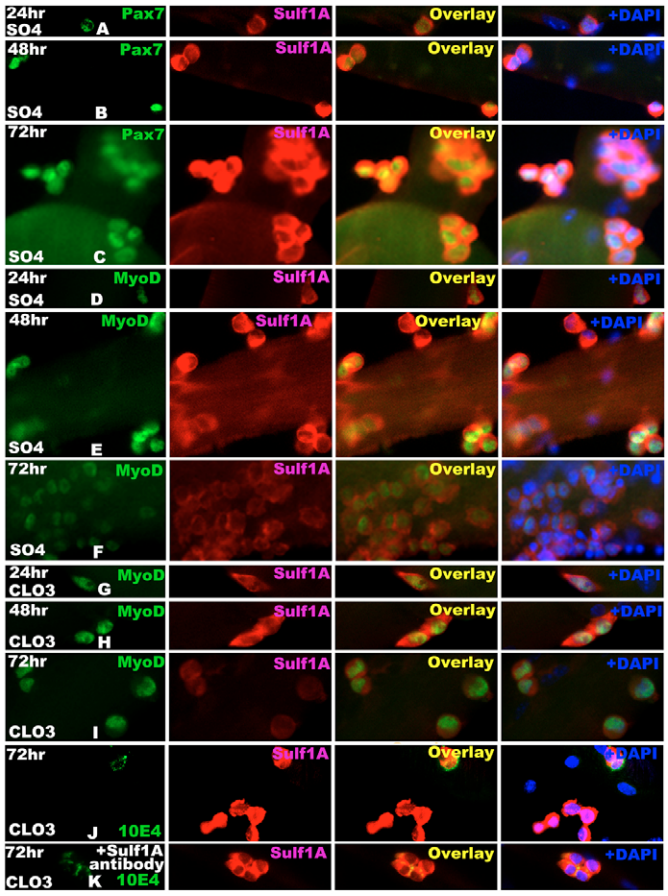

Fig. 3. Increased chemical sulfation results in a moderate increase in satellite-cell proliferation whereas chemical de-sulfation results in a slight decrease. Satellite cells on single fibres grown in culture medium containing 8 mM sodium sulfate (**A-F**) or 8 mM sodium chlorate (**G-J**), or 8 mM sodium chlorate+100 ng/ml Sulf1A antibody, stained after 24, 48 and 72 hours for Pax7 (column1 A-C) or for MyoD (column 1 D-I) or for sulfation with antibody 10E4 (J, **K**) and Sulf1A (column 2) using a double immunofluorescence procedure. The superimposed images of Pax7 and Sulf1A or MyoD and Sulf1A or 10E4 and Sulf1A staining are shown in column 3 with additional DAPI staining in column 4. CLO3, chlorate; SO4, sulfate. Anti-Sulf1A antibody was used at a concentration of 100 ng/ml.

ng/ml anti-Sulf1A antibody to neutralise Sulf1A activity in such cultures did not overcome chlorate-induced de-sulfation (Fig. 3K).

Reduced de-sulfation using Sulf1A-neutralising antibodies enhances satellite-cell proliferation leading to Pax7 and MyoD downregulation

Since 8 mM chlorate appeared to reduce satellite cell proliferation, although only slightly, we tried to reduce Sulf1A activity by exposing the satellite cells on single fibres to Sulf1A-neutralising antibodies at different concentrations. One of the Sulf1A antibodies (antibody B) was added to the culture medium at 25, 50 and 100 ng/ml at time 0 (Fig. 4A-F) whereas the second Sulf1 antibody was added only at 100 ng/ml concentration (Fig. 4G,H), because concentrations of 50-100 ng/ml produced optimal neutralisation as judged by immunocytochemical characterisation of satellite-cell progeny (see below). Sulf1A and MyoD expression levels in all such satellite cells on dissociated single fibres in vitro were investigated after 24 and 72 hours. Despite the addition of Sulf1A antibodies to reduce Sulf1A activity, Sulf1A was still readily apparent in most satellite cells in all the different concentration treatments (Fig. 4). The addition of only 25 ng/ml Sulf1A antibodies, however, did not result in any significant quantitative or qualitative change in either the satellite-cell proliferation or expression levels of either Sulf1A or MyoD at 24 or 72 hours (Fig. 4). By contrast, increasing the Sulf1A antibody concentration from 25 ng to 50 or 100 ng/ml markedly increased satellite-cell proliferation when compared with control cultures at 72 hours, although no effect was apparent at 24 hours (Figs 4, 7). In addition to increased satellite-cell proliferation, the exposure to Sulf1A antibodies at both 50 and 100ng/ml also produced marked downregulation of MyoD in a large proportion of the satellite cells at 72 hours (Figs 4, 7).

The neutralisation of some Sulf1A by Sulf1 antibodies was confirmed by increased sulfation levels of antibody-treated satellite cells (Fig. 5A-C) observed using immunocytochemical staining with antibody 10E4 that stains HS, but not following desulfation leading to removal of this epitope (Fuxe et al., 1994). Sulf1,

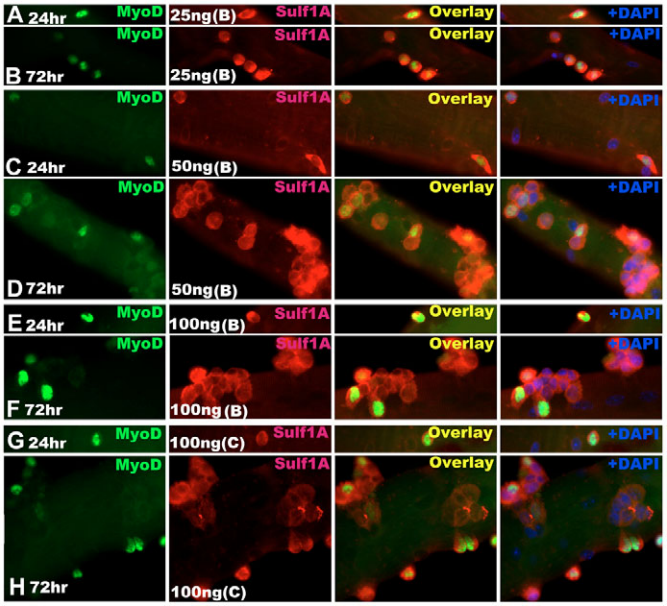

Fig. 4. Sulf1A-neutralising antibodies increase satellite-cell proliferation and MyoD downregulation. Satellite cells on single fibres grown in culture medium containing Sulf1A antibody B at 25 ng/ml (**A**,**B**), 50 ng/ml (**C**,**D**), 100 ng/ml (**E**,**F**), or Sulf1 antibody C for total Sulf1 at 100 ng/ml (**G**,**H**) stained after 24 (A,C,E,G) or 72 hours (B,D,F,H) for MyoD (column 1) and Sulf1A (column 2) using a double immunofluorescence procedure. The superimposed images of MyoD and Sulf1A staining are shown in column 3 with additional DAPI staining in column 4; hr, hours.

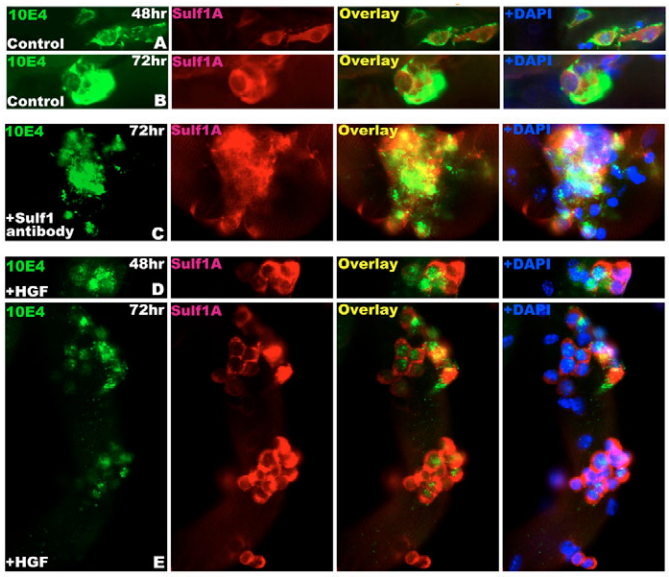

Fig. 5. Relationship of Sulf1A activity to sulfation levels under different growth conditions. Satellite cells on single fibres grown in normal growth medium (**A**,**B**), or medium containing either 100 ng/ml Sulf1A antibody (**C**) or 100 ng/ml HGF (**D**,**E**), stained after 48 or 72 hours to examine sulfation levels with antibody 10E4 (column 1) in relation to the Sulf1A expression pattern (column 2), using a double immunofluorescence procedure. The superimposed images (overlay) are shown in column 3 with the addition of DAPI staining in column 4; hr, hours.

however, removes only 6-O sulfates, leaving all other sulfate moieties intact on HS and could thus still show some positive reaction with antibody 10E4 due to its binding to those sulfates. One also needs to distinguish between the intracellular and membrane staining, the site of growth factor signalling. Despite all these limitations, the staining of control satellite cells with antibody 10E4 showed variable levels of sulfation in some satellite cells at different stages of development (Fig. 5A,B). A higher level of sulfation on the cell membrane, however, was observed in a large majority of the control satellite cells although some larger cell clusters also showed high levels of cytoplasmic sulfation. In comparison with the control, treatment of satellite cells with Sulf1A antibodies also showed variable but generally higher level of sulfation, indicating some Sulf1A neutralisation or reduced Sulf1A expression (Fig. 5C). The change in satellite-cell growth, presumably due to Sulf1A neutralisation and/or reduction, however, was clearly apparent, not only from the increased satellite-cell cluster size but also downregulation of MyoD in a large proportion of the satellite cells (Fig. 7) that appeared more akin to HGF treatment (see below).

HGF markedly enhances satellite-cell proliferation characterised by MyoD and Pax7 downregulation

HGF, present in an inert form in normal adult skeletal muscle is known to be activated upon injury (Tatsumi and Allen, 2004) to drive satellite-cell activation and proliferation. HGF enhanced satellite-cell proliferation and downregulated both MyoD and Pax7 (see Figs 6 and 7). Since HGF, like the FGFs, is known to require sulfated HSPGs as secondary receptors, Sulf1A expression levels could regulate HGF activity. We therefore investigated the expression pattern of Sulf1A in activated satellite cells upon increased exposure to HGF in vitro. This study demonstrated that although Sulf1A could inhibit HGF activity, its expression in satellite cells was nevertheless observed throughout the HGF exposure period (Fig. 6). The levels of Sulf1A expression, however, varied considerably in individual satellite cells at different stages (Fig. 6) with Sulf1A, persisting in a large majority of the satellite cells throughout the 72-hour culture period. In fact, Sulf1A levels in a large proportion of the satellite cells appeared particularly high at 72 hours when compared with 24 and 48 hour incubations. At 48 and 72 hours, Sulf1A expression was generally higher in Pax7- and MyoD-negative satellite cells (Fig. 6). One of the marked features of HGF exposure, however, was the relatively earlier and greatly enhanced downregulation of both Pax7 and MyoD in a large majority of the satellite cells as indicated by immunocytochemical staining at both 48 and 72 hours (Figs 6, 7).

HGF-treated satellite cells also demonstrated highly variable levels of sulfation, as revealed by staining with the antibody 10E4 (Fig. 5D,E). Although some high Sulf1A-expressing satellite cells demonstrated a reciprocal relationship with 10E4 antibody staining, i.e. lack of sulfation as expected, other satellite cells within the same cell clusters did not exhibit such a tight reciprocal relationship, indicating a high level of diversity in sulfation levels of satellite cells that may relate to quiescence or HGF-induced proliferation to different extents within different subsets of satellite cells (Fig. 5D,E).

Further characterisation of Sulf1A^{+}-Pax7^{-} and Sulf1A^{+}-MyoD^{-} satellite-cell sub-populations

A large number of satellite cells grown in the presence of HGF or antibodies to Sulf1A that downregulated MyoD and or Pax7

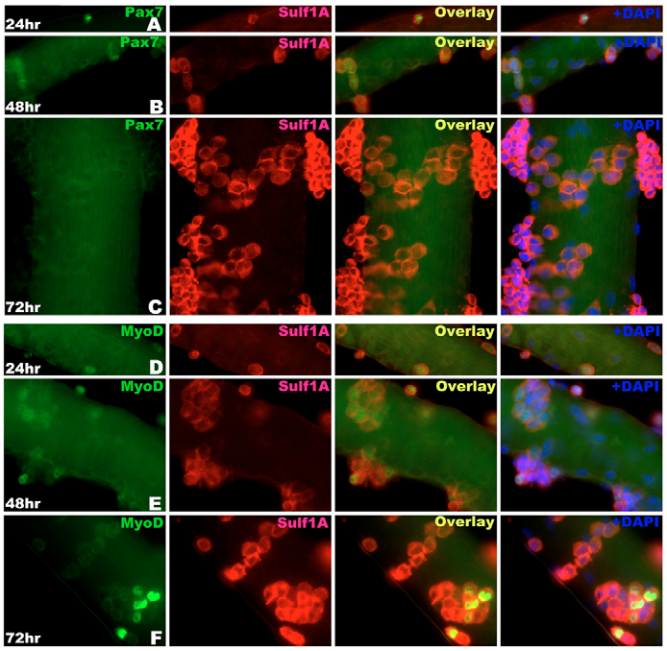

Fig. 6. HGF enhances satellite-cell proliferation accompanied by MyoD and Pax7 downregulation. Satellite cells on single fibres grown in growth medium containing 100 ng/ml HGF stained after 24, 48 and 72 hours for Pax7 (**A-C**, column 1) or MyoD (**D-F**, column 1) and Sulf1A (column 2) using a double immunofluorescence procedure. The superimposed images of Pax7 and Sulf1A or MyoD and Sulf1A staining are shown in column 3 with additional overlay of DAPI staining in column 4. hr, hours.

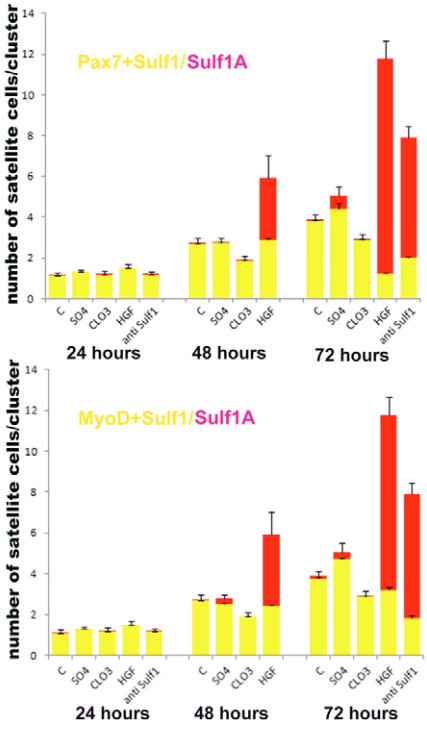

Fig. 7. The quantification of satellite cells per cluster and pattern of MyoD, Pax7 or Sulf1A expression under different culture conditions. The quantification of satellite cells per cluster under normal culture conditions (C) and when 8 mM sodium sulfate (SO4) or 8 mM sodium chlorate (CLO3) or 100 ng/ml HGF or 100 ng/ml Sulf1A antibodies were added to the culture medium for 24, 48 or 72 hours. The total height of each bar represents the number of satellite cells per cluster, whereas the height of the yellow bar represents the number of satellite cells per cluster staining for Sulf1A+Pax7 (top panel) or Sulf1A+MyoD (bottom panel). Red bars indicate the cells stained for Sulf1A but not Pax7 or MyoD. The addition of both HGF and anti-Sulf1A antibody induced a significant increase in the size of the satellite-cell cluster at 72 hours when compared with control, HGF (P<0.001), anti-Sulf1A (P<0.03), control (P<0.001). Subtle changes at 24 hour were not statistically significant, but with HGF inclusion producing a significant change at 48 hours (P<0.002). The downregulation of Pax7 and MyoD in a large proportion of the Sulf1A$^+$ satellite cells at 72 hours was also statistically significant, Pax7 (P<0.002), MyoD (P<0.01) as it was also at 48 hours Pax7 (P<0.03), MyoD (P<0.02).

expression (Figs 5, 6) usually did not induce myogenin expression (Fig. 8) but expressed both Myf5 and Sulf1A although Myf5 was undetectable in quiescent satellite cells (Fig. 8). The transfer of such satellite-cell clusters to gelatin-coated culture dishes showed all cells to re-activate MyoD (Fig. 9) and Pax7 (not shown). Virtually all satellite-cell progeny from not only control cultures but also HGF-treated and Sulf1A-antibody-treated cultures expressed MyoD at 72 hours although the levels of this transcription factor varied amongst cells (Fig. 9).

To determine whether satellite-cell progeny at 72 hours after exposure to anti-Sulf1A or HGF were progressing towards a quiescent or proliferative phenotype, expression of proliferating cell nuclear antigen (PCNA), a protein synthesised in early G1 and S phases of the cell cycle, was examined as well as expression of myogenin and Sulf1A. The PCNA antibody stained virtually all satellite cells (97±0.52%) in control culture medium at 72 hours whereas the number of cells staining with this antibody in both HGF- and anti-Sulf1A-treated cultures (Fig. 10) was much smaller (21.6±2.55%). The PCNA staining was observed in both myogenin$^+$ (yellow cells in overlay, Fig. 10) and myogenin$^-$ cells (green cells in overlay, Fig. 10) in both control and anti-Sulf1A-treated cultures at 72 hours. The proliferation status of these cells was further confirmed by immunocytochemical staining for phospho-histone H3 (not shown), another marker of proliferation, showing patterns of expression similar to PCNA. Pulsing of single-fibre cultures with 10 μM BrdU at 48 hours followed by BrdU immunocytochemical investigation at 72 hours (not shown) also confirmed a much lower number of proliferating cells in HGF- and anti-Sulf1A-treated satellite-cell cultures at 72 hours.

Discussion

Sulf1A expression was undetectable in adult muscle fibres as well as Pax7-positive quiescent satellite cells on isolated single fibres although this enzyme is expressed in myogenic cells during early muscle development (this study) (Dhoot et al., 2001). Sulf1A, however, was re-activated in regenerating myotubes following experimental as well as spontaneous muscle regeneration in mdx mouse dystrophic muscle, thus re-capitulating early muscle development in which it regulates a number of growth factors or signalling molecules implicated during early phase of muscle development and regeneration. The importance of Sulf1A in myogenesis was also indicated by our earlier study in which Sulf1A inhibition by antisense oligonucleotides led to downregulation of MyoD (Dhoot et al., 2001) in newly generated somites. The present study also demonstrates earlier activation of Sulf1A than MyoD in in vitro satellite cells, not only during the 9- to 12-hour period

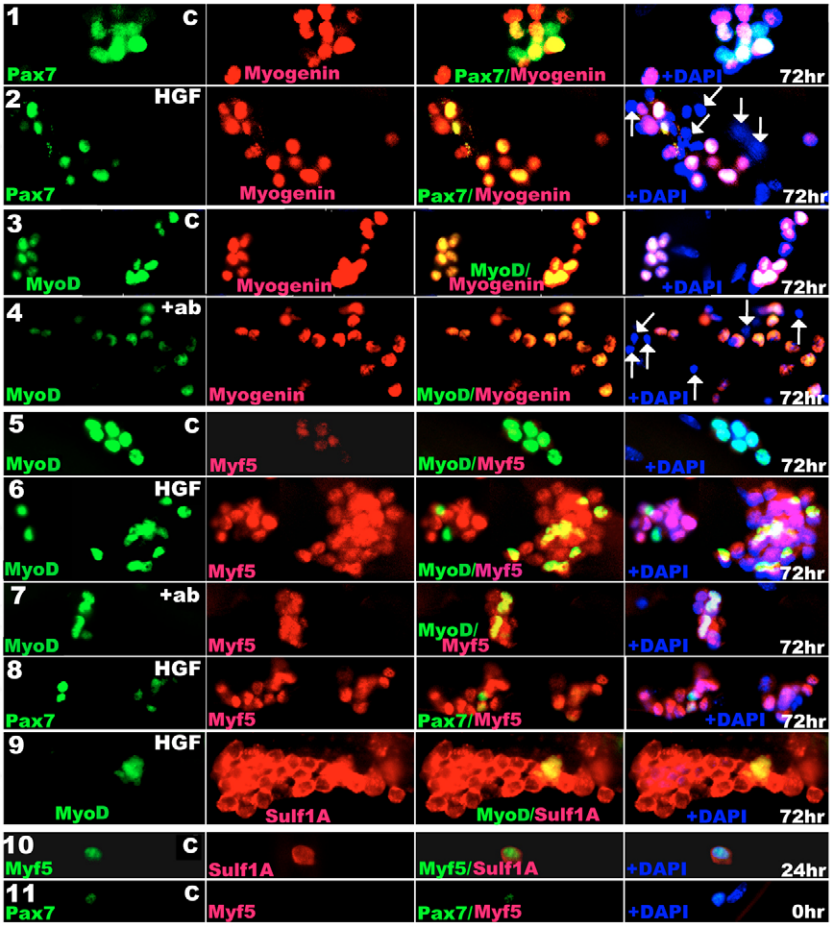

Fig. 8. Further characterisation of control and Pax7$^-$ and or MyoD$^-$ cells in HGF and Sulf1A-antibody-treated cultures. A proportion of satellite cells on single fibres that cultured in the presence of HGF (rows 2, 6, 8 and 9) or Sulf1A antibody (rows 4 and 7) following 72 hours in culture, downregulated MyoD and Pax7 without activating myogenin expression but did express Sulf1A and Myf5. Myf5 was undetectable in quiescent satellite cells (row 11) but was activated by in vitro culture (row 10). The expression of Pax7, MyoD, Myf5, myogenin (column 1) and Sulf1A (column 2) is shown individually as well as superimposed images (column 3) and with DAPI (column 4), under different culture conditions. The satellite cells that had downregulated both Pax7 and myogenin in the presence of HGF (row 2) or MyoD and myogenin in the presence of Sulf1A antibody (row 4) are only apparent by blue DAPI staining of their nuclei (white arrows). Myf5 expression in such cultures was usually detectable not only in MyoD$^-$ and Pax7$^-$ cells but also in a sub-population still expressing these transcription factors. C, control; HGF, hepatocyte growth factor at 100 ng/ml; +ab, + 100 ng/ml Sulf1A antibody; hr, hours.

when it could relate to the relative avidities of the Sulf1A and MyoD antibodies but also during the later stage of 24-30 hours, when some Sulf1A-positive satellite cells within the same doublet or triplet were still MyoD negative despite intense MyoD staining of some satellite cells within the same cluster. Sulf1A expression therefore may have a role in triggering MyoD activation. However, all satellite cells, under normal culture conditions, were found to be both Sulf1A and MyoD positive at 48 hours in vitro, whereas downregulation of MyoD was observed in a small number of satellite cells at 72 hours. This could indicate the onset of differentiation or divergence into a separate sub-population (Seale et al., 2004; Halevy et al., 2004). Variable Sulf1A expression levels at 72 hours could have a selective role in myogenic regulation through its regulation of growth factor signalling.

This study confirms earlier investigations demonstrating Pax7 expression in quiescent satellite cells and during activation and proliferative satellite states, but downregulation during differentiation with the onset of myogenin expression (Zammit et al., 2002; Zammit et al., 2004; Halevy et al., 2004; Olguin et al., 2007). The number of satellite cells showing downregulation of Pax7 in control cultures in the present study, however, was much lower, although the level of Pax7 expression had decreased in a large number of cells. The increased sulfation levels following the addition of 8 mM sodium sulfate to the culture medium led to a modest increase in satellite-cell proliferation. This may result from increased activities of some FGFs and HGF (Villena and Brandan, 2004) present in the normal medium supplemented with foetal calf serum and embryo extract, but no apparent change in the pattern of Pax7 expression. The global desulfation by the addition of 8 mM sodium chlorate slightly decreased satellite-cell proliferation but did not result in any change in MyoD or Pax7 expression. Sulf1A expression, as judged by immunocytochemical staining during the 72-hour culture period, however, did not appear to be inhibited during chemically increased or decreased levels of sulfation. Therefore, chemical desulfation or sulfation at the levels that we used in this study did not markedly alter proliferation or differentiation characteristics of satellite cells although proliferation in chlorate cultures was slightly reduced. This is compatible with studies reported by Cornelison et al. (Cornelison et al., 2001) who demonstrated reduction in satellite-cell number using 25 mM chlorate which delayed proliferation but did not affect migration or apoptosis. Chlorate-induced global desulfation of HS is not strictly similar to specific removal of 6-O sulfates by Sulf1A as different sulfate moieties have specific and distinct roles in different stages of growth factor signalling. Furthermore, chemical reduction or increase in sulfation at specific concentrations may not alter sulfation levels optimal for HGF and/or FGF or Wnt signalling. A more specific and subtle change in sulfation level was therefore induced by Sulf1A inhibition using Sulf1A neutralising antibodies. Although low concentration of Sulf1A antibodies, at 25 ng/ml, produced no significant effect on either the proliferation or MyoD expression pattern, satellite-cell proliferation was enhanced by increasing the antibody levels in the growth medium to 50 or 100 ng/ml. Enhanced satellite-cell

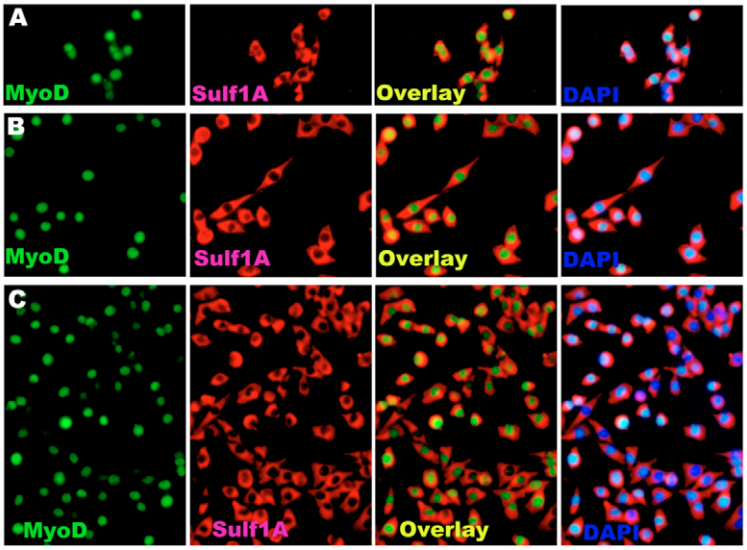

Fig. 9. All satellite-cell-derived cells, including Pax7$^-$ or MyoD$^-$ cells, apparent in HGF-treated and Sulf1A-antibody-treated cultures, re-activate MyoD expression upon sub-culture in fresh growth medium on gelatin-coated dishes. Satellite cells grown on single fibres for 72 hours in normal culture medium (**A**), or the growth medium containing 100 ng/ml Sulf1A antibody (**B**) or 100 ng/ml HGF (**C**) were transferred for sub-culture onto gelatin-coated dishes containing 10% FCS (fresh medium) for a further 72 hours before immunocytochemical staining for MyoD (column 1) or Sulf1A (column 2). The superimposed images are shown in column 3 and a further overlay with DAPI in column 4.

proliferation also became apparent at 50 and 100 ng/ml, which resulted in accelerated downregulation of MyoD in satellite-cell progeny. The application of Sulf1A antibodies to the culture medium thus affected satellite-cell growth as the number of satellite cells per cluster increased considerably although it required at least 50 ng/ml to elicit a growth-enhancing response. Although Sulf1A neutralisation with Sulf1A antibodies promoted satellite-cell proliferation, significant Sulf1A expression was still detectable in antibody-treated cells at both 24 and 72 hours. The observed Sulf1A levels may persist because of further stimulation of Sulf1A in response to the Sulf1A reduction or because of exogenously supplied bound Sulf1A antibodies. This could result in Sulf1A visualisation by immunocytochemical staining without Sulf1A activity. Therefore, the level of Sulf1A activity in such treated cultures was further examined using antibody 10E4 that demonstrated reduced Sulf1A activity resulting in reduced desulfation. The increased satellite-cell proliferation in Sulf1A-antibody-treated cultures could thus be related to enhanced FGF and/or HGF signalling, resulting from the sulfation levels being optimal for such growth factor signalling. Residual Sulf1A levels, nevertheless, did not inhibit growth factor activities required to promote satellite-cell proliferation. Sulf1A activity may be essential to control regulated proliferation ensuring timely progression to differentiation by repressing FGF and HGF signalling in satellite cells.

Pax7 and MyoD expression persisted in most satellite cells on single fibres cultured in normal growth medium for up to 72 hours but there was a rapid downregulation of both these transcription factors at 72 hours, and to a lesser extent at 48 hours, in HGF-treated cultures and in the presence of Sulf1A-neutralising antibodies. This resulted in a marked heterogeneity within cell clusters with some cells still staining for Pax7 or MyoD while a large number of cells downregulated the expression of both these transcription factors although still expressing Sulf1A. MyoD expression in some HGF-treated cultures had already been repressed in a subset of cells to almost an undetectable level at 48 hours when virtually all satellite cells at this stage are usually positive for MyoD under control culture conditions. Pax7 and MyoD downregulation is usually believed to indicate onset of differentiation, as MyoD is replaced with myogenin, but a number of Pax7$^-$ and MyoD$^-$ cells in both HGF- and neutralising-antibody-treated cultures in the present study did not show myogenin activation and therefore represented a distinct sub-population of satellite cells. Further characterisation of this MyoD$^-$/Pax7$^-$/Sulf1$^+$ satellite-cell sub-population showed that these cells did not only repress myogenin activation but activated Myf5 instead (Yamane

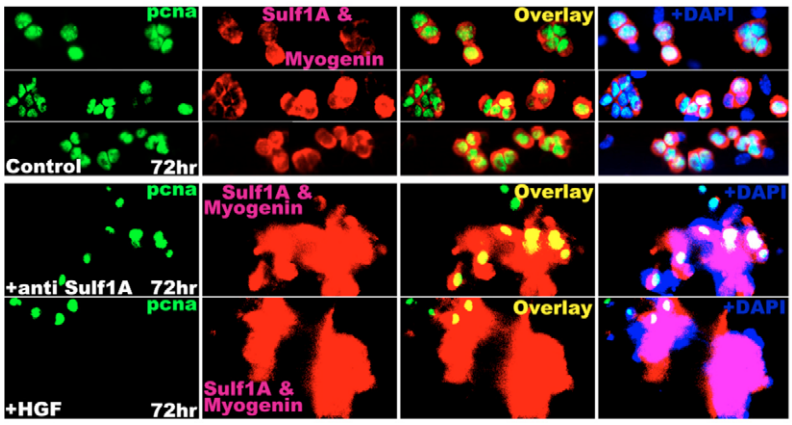

Fig. 10. The proportions of PCNA-positive (proliferating) cells in control and anti-Sulf1A- or HGF-treated satellite-cell cultures at 72 hours. Isolated single fibres grown in normal culture medium or medium +100 ng/ml HGF or 100 ng/ml Sulf1A antibody for 72 hours, stained for PCNA (green nuclei) or myogenin (red nuclei) + Sulf1A (red cytoplasmic and membrane staining) and with DAPI (blue) showing total nuclei. Overlays show PCNA+myogenin in nuclei as yellow while the presence of PCNA alone without myogenin appears green.

et al., 2004), an early myogenic marker that is undetectable in quiescent satellite cells but is activated before MyoD during embryonic myogenesis (Tajbakhsh and Buckingham, 1994). These cells thus still belong to the myoblast/satellite-cell lineage with Myf5 expression in a large majority of the cells. The phenotype of this sub-population was further confirmed by re-activation of MyoD in all such cells when sub-cultured on gelatin-coated culture dishes thus confirming their myogenic lineage. It would, however, require further characterisation and sub-culture studies to establish if this sub-population exhibits different proliferative or some stem-cell-like characteristics. The PCNA antibody staining of HGF- and anti-Sulf1A-treated cultures demonstrated reduction of $PCNA^+$ cells in these cultures compared with control cultures at 72 hours, indicating cell cycle arrest by this time despite much greater multiplication during earlier stage. This could also indicate rapid growth factor exhaustion in treated cultures compared with controls or the physical constraints of a limited number of satellite cells per cluster. This sub-population thus requires further characterisation using multiple markers. The emergence of different subsets of myogenic cells has also been reported in some other studies. For example, Yoshida et al. (Yoshida et al., 1998) demonstrated downregulation of MyoD expression in a subset of mouse C2C12 cells. Differences in MyoD expression have been shown to determine whether a myogenic cell would differentiate by activating myogenin synthesis or be put aside as a reserve cell. In this study we demonstrate that not only MyoD downregulation but also Pax7 downregulation is observed in a subset of satellite-cell progeny although the identity of this sub-population with known satellite-cell lineages requires further characterisation. This study thus demonstrated that after several cell division cycles, $Pax7^+/MyoD^+$ cells underwent asymmetric cell division to generate both $Pax7^+/MyoD^+$ and $Pax7^-$ and or $MyoD^-$ cells, the number of which was considerably increased in the presence of HGF and Sulf1A-neutralising antibodies. The significance of Pax7 downregulation without myogenin activation is not clear, but a recent study by Lepper et al. (Lepper et al., 2009) has demonstrated that unlike embryonic muscle, lack of Pax7 in adult satellite cells does not compromise adult muscle regeneration. Pax7 re-expression in such cells may also occur after Myf5 downregulation at a later stage.

What activates quiescent satellite cells into a proliferative state is still far from clear, although injury to muscle is believed to release some diffusible growth factors such as HGF and FGF family members, which are stored in an inert form in the basal lamina, to trigger satellite-cell activation (Bischoff, 1986; Tatsumi et al., 1998; Yablonka-Rueuveni et al., 1999; Tatsumi and Allen, 2004; Chargé and Rudnicki, 2004). HGF is the major contributing factor as this activity is abolished by pre-incubation with HGF-neutralising antibodies. HGF is an autocrine factor synthesised and released during the period of rapid proliferation and subsequent self-renewal. HGF signalling requires the availability of the HGF tyrosine kinase receptor called c-met that is localised in satellite cells. As is the case with FGFs, HGF also requires sulfated HSPGs as secondary receptors for cell signalling (Cornelison et al., 2001; Derksen et al., 2002). HGF activity is inhibited by Sulf1 action (Lai et al., 2004) as it removes 6-O sulfates from HS, and yet Sulf1A was found to be expressed in a large majority of the satellite cells throughout the 72-hour HGF exposure period. Despite some Sulf1A expression, HGF was clearly functioning at a sufficiently high level to greatly enhance satellite-cell proliferation, as indicated by greatly increased satellite-cell cluster size at both 48 and 72-hours. Furthermore, a greatly enhanced HGF activity was also apparent from rapid downregulation of both Pax7 and MyoD in a large proportion of the satellite-cell progeny even at 48 hours in vitro. It is, however, not only the activities of HGF and FGFs that contribute to the satellite-cell activation and proliferation but also canonical Wnt signalling (Otto et al., 2008) that would be enhanced by Sulf1A activity. Sulf1A expression therefore may be related to spatially and temporally associated or dissociated canonical Wnt signalling since it is the net balance of multiple activities that regulates the overall satellite-cell proliferation and differentiation characteristics. Sulf1A could thus regulate satellite-cell proliferation and differentiation by both negative and positive regulation of HGF and/or FGF and Wnt signalling activities. We conclude that the level of Sulf1A activity has a key role not only in muscle development but also muscle regeneration by regulating satellite-cell activity in injured or diseased muscle.

Materials and Methods

In situ hybridisation procedure

The extensor digitorum longus (EDL) muscles from 27-day-old mdx mice were fixed overnight in 4% paraformaldehyde at 4°C before being embedded in paraffin wax and sectioned at 10 or 6 μm thickness for in situ hybridisation and immunocytochemical analyses. After wax removal, an reverse transcriptase (RT)-PCR-generated digoxigenin-labelled 292 bp riboprobe (3637) to mouse Sulf1 (1686-1978 bp) was used to analyse *Sulf1* mRNA in 10 μm thick sections using the in situ hybridisation procedure described by Moorman et al. (Moorman et al., 2001).

Myofibre isolation and culture

EDL muscles were carefully dissected from 8- to 10-week-old CD mice killed using Home-Office-approved Schedule 1 procedure. Each dissected muscle was transferred to a bijou containing 1 ml 0.12% Type 1 collagenase (Sigma) in DMEM. Isolated muscles in collagenase solution were incubated at 37°C for 2-3 hours with light shaking every 15 minutes, until the muscle fibres had dissociated. Once dissociated, the collagenase activity was inhibited by the addition of 10% FCS-DMEM before single-fibre selection was carried out under a Leica stereo microscope and 12-15 single fibres transferred into each of the wells of a 24-well Linbro plate with 0.5 ml/well culture medium. The normal culture medium was composed of DMEM (Gibco), 4 mM L-glutamine (Sigma), 1% penicillin-streptomycin (Sigma), 10% foetal calf serum (FCS) and 0.5% chick embryo extract (MP Biomedicals). The addition of other components, such as 8 mM sodium sulfate or 8 mM sodium chlorate or 100 ng/ml HGF or Sulf1A antibodies at 25, 50 or 100 ng/ml concentration, to the culture the media in some experiments was carried out at time 0. The dissociated single muscle fibres suspended in culture medium were incubated at 37°C, 5% CO_2 before fixation in 4% paraformaldehyde for 15 minutes after different time intervals, which are listed in the Results section. Some muscle fibres were also fixed at time 0 to investigate the quiescent satellite-cell phenotype.

For adherent cultures, isolated single fibres from controls and cultures treated with antibodies to Sulf1A and HGF following 72 hours in vitro were transferred to four-well gelatin-coated dishes and cultures maintained in 10% FCS at 37°C in 5% CO_2. Following a further 72 hours in vitro growth on gelatin-coated four-well dishes, single fibres were removed before fixation of adherent cells on the dishes in 4% paraformaldehyde for 15 minutes for immunocytochemical staining with antibodies to Pax7 or MyoD and Sulf1A.

Regenerating muscle

Frozen sections of gastrocnemius muscles of 3-week-old chicks crush injured at 2 weeks of age, and allowed to recover for 7 days post-injury as described in our earlier study (Zhang and Dhoot, 1998) were used for immunocytochemical staining for Sulf1A and striated muscle myosin heavy chain expression. In addition, paraffin sections of spontaneously regenerating mdx muscles were used for analysis of Sulf1A expression as described above.

Immunocytochemical procedure

Different polyclonal and monoclonal antibodies were used to study the expression patterns of Sulf1A, Pax7 and MyoD. For Sulf1A expression, a Sulf1A-specific rabbit polyclonal antibody (B) raised to an 18-amino-acid peptide in the hydrophilic domain was used for most analyses although another Sulf1 antibody (C) raised to a 20-amino-acid peptide present in the C-terminal domains of Sulf1 was also used in some experiments as stated in the Results section. The specificities of Sulf1 antibodies, established immunochemically using immunoblotting and immunocytochemical procedures, have been described previously (Sahota and Dhoot, 2009). Pax7, MF20 myosin heavy chain and F5D myogenin mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank; anti-MyoD mouse monoclonal (clone 5.8A) antibody was obtained from DakoCytomation; and a mouse monoclonal antibody (PC10) for proliferating cell nuclear antigen (PCNA), was purchased from

Santa Cruz. Santa Cruz Myf5 antibody (sc-302) was kindly provided by Michelangelo Campanella (Rossi et al., 2010), and 83B6 mouse monoclonal antibody (Dhoot et al., 1986) was used to stain for total striated muscle myosin heavy chains in some sections as it reacts with all isoforms.

The fixed single fibres for PCNA antigen retrieval were incubated in 10 mM citrate buffer for 20 minutes at 95°C before staining with PC10 antibody. For immunocytochemical staining, fixed muscle fibres or tissue sections were washed with PBS and permeabilised for 15 minutes using a buffer composed of 20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton X-100 (pH 7). Muscle fibres were then washed in PBS and nonspecific antibody binding blocked using 10% FCS in PBS for 30 minutes before incubation with the primary antibodies. All primary antibody reactions were carried out overnight at 4°C for approximately 16 hours followed by secondary antibody incubations for 1 hour each, at room temperature. All primary and secondary antibodies were diluted in 10% FCS. Sulf1 primary antibodies were diluted 1/200, Pax7 supernatant antibody 1/4, MyoD antibody 1/150, F5D supernatant antibody 1/100, Myf5 antibody (sc-302) 1/50, 83B6 antibody 1/100, MF20 supernatant antibody 1/10, PC10 antibody 1/200; all fluorochrome-labelled secondary antibodies were diluted 1/300. Antibody binding by mouse monoclonal antibodies was usually detected using Alexa Fluor 488 fluorochrome-conjugated goat anti-mouse IgG (Molecular Probes, A11029) and Sulf1 rabbit primary antibodies were visualised using goat anti-rabbit biotinylated IgG (DAKO, E0353) followed by streptavidin-conjugated Alexa Fluor 594 (Molecular Probes S11227). In case of double immunofluorescence using two different mouse monoclonal antibodies, for example MyoD and myogenin or Pax7 and myogenin, the staining was performed sequentially so that the first mouse monoclonal antibody was always probed with Alexa-Fluor-488-conjugated goat anti-mouse IgG followed by incubation with second mouse monoclonal, probed with Alexa-Fluor-594-conjugated goat anti-mouse IgG. Single fibres or tissue sections were mounted using fluorescent mounting medium (Sigma-Aldrich) containing 2.5 μg/ml DAPI for nuclear visualisation and photographed using a Leica DM4000B fluorescence microscope.

For quantification, the total number of satellite cells per cluster for each category at 24, 48 and 72 hours was counted from Sulf1A antibody staining as this antibody at these time points stained most satellite cells. The number of satellite cells staining for Pax7 or MyoD per cluster was also counted from double immunofluorescence staining for Pax7 or MyoD combined with Sulf1 antibodies. A minimum of 30 cell clusters from at least 15 different muscle fibres were counted for each group. The data from multiple clusters were pooled to obtain a mean (± s.e.m.) for each category. Data are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA, regarding $P<0.05$ as statistically significant.

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