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ADAM12 and $\alpha_9\beta_1$ Integrin Are Instrumental in Human Myogenic Cell Differentiation

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Knowledge on molecular systems involved in myogenic precursor cell (mpc) fusion into myotubes is fragmentary. Previous studies have implicated the *a d*isintegrin *a*nd *m*etalloproteinase (ADAM) family in most mammalian cell fusion processes. ADAM12 is likely involved in fusion of murine mpc and human rhabdomyosarcoma cells, but it requires yet unknown molecular partners to launch myogenic cell fusion. ADAM12 was shown able to mediate cell-to-cell attachment through binding $\alpha_9\beta_1$ integrin. We report that normal human mpc express both ADAM12 and $\alpha_9\beta_1$ integrin during their differentiation. Expression of α_9 parallels that of ADAM12 and culminates at time of fusion. α_9 and ADAM12 coimmunoprecipitate and participate to mpc adhesion. Inhibition of ADAM12/$\alpha_9\beta_1$ integrin interplay, by either ADAM12 antisense oligonucleotides or blocking antibody to $\alpha_9\beta_1$, inhibited overall mpc fusion by 47–48%, with combination of both strategies increasing inhibition up to 62%. By contrast with blockade of vascular cell adhesion molecule-1/$\alpha_4\beta_1$, which also reduced fusion, exposure to ADAM12 antisense oligonucleotides or anti-$\alpha_9\beta_1$ antibody did not induce detachment of mpc from extracellular matrix, suggesting specific involvement of ADAM12–$\alpha_9\beta_1$ interaction in the fusion process. Evaluation of the fusion rate with regard to the size of myotubes showed that both ADAM12 antisense oligonucleotides and $\alpha_9\beta_1$ blockade inhibited more importantly formation of large (≥5 nuclei) myotubes than that of small (2–4 nuclei) myotubes. We conclude that both ADAM12 and $\alpha_9\beta_1$ integrin are expressed during postnatal human myogenic differentiation and that their interaction is mainly operative in nascent myotube growth.

INTRODUCTION

Adult skeletal muscle regeneration after injury results from activation, proliferation, and fusion of mononucleated myogenic precursor cells (mpc) (Hawke and Garry, 2001). The mpc fusion results from an ordered sequence of events, including clustering and alignment of cells, establishment of close cell-to-cell contacts, and plasma membrane merging (Doberstein *et al*., 1997; Taylor, 2003). Various membrane proteins have been implicated in myotube formation, including N- and M-cadherins, neural cell adhesion molecule (NCAM), and vascular cell adhesion molecule (VCAM), $\alpha_4\beta_1$ and other integrins, and *a d*isintegrin *a*nd *m*etalloproteinases (ADAMs) (Abmayr *et al*., 2003). ADAMs form a family of >30 transmembrane glycoproteins with a unique domain organization, including a prodomain, a proteolytic domain (metalloprotease), an adhesion integrin-binding site formed by both disintegrin and cysteine-rich domains, an epidermal growth factor-like domain, a transmembrane domain, and a signaling cytoplasmic tail (Huovila *et al*., 1996; Primakoff and Myles, 2002; White, 2003). In addition, some ADAMs contain a hydrophobic sequence in a cysteine-rich region that may represent a fusion peptide, suggesting that this subclass of ADAMs might participate to plasma membrane merging (Huovila *et al*., 1996). Some ADAMs have been implicated in most mammalian cell fusion processes, including ADAM1, 2, and 3 in fertilization (Blobel *et al*., 1992; Huovila *et al*., 1996; Wolfsberg and White, 1996; Hooft, 1998) and ADAM12 in osteoclast (Abe *et al*., 1999; Choi *et al*., 2001) and macrophage-derived multinucleated giant cell formation (Abe *et al*., 1999), in trophoblast syncytialization (Huovila *et al*., 1996; Gilpin *et al*., 1998; Shi *et al*., 2000), and in myogenesis (Yagami-Hiromasa *et al*., 1995).

Several ADAMs are expressed by adult and developing skeletal muscles, including ADAM1, 4, 9, and 15 that are ubiquitous, and ADAM12, also called meltrin-α, whose expression is less widespread (Yagami-Hiromasa *et al*., 1995; Loechel *et al*., 2000; Kratzschmar *et al*., 1996; Weskamp *et al*., 1996; Kurisaki *et al*., 1998). In rodents, constitutive muscle expression of ADAM12 starts at the embryonic stage when myotubes are formed (Kurisaki *et al*., 1998), persists at the neonatal stage (Yagami-Hiromasa *et al*., 1995; Borneman *et al*., 2000; Kronqvist *et al*., 2002), and ceases in adulthood (Borneman *et al*., 2000; Kronqvist *et al*., 2002). Adult muscle regeneration after experimental injury is associated with ADAM12 reexpression by fusing myogenic cells and newly formed fibers (Galliano *et al*., 2000; Kronqvist *et al*., 2002). Consistently, ADAM12 expression parallels fusion and myogenin expression of C2C12 myogenic cells (Yagami-Hiromasa *et al*., 1995). Transfection experiments have demonstrated that the recombinant disintegrin and cysteine-rich domains of ADAM12 are crucially involved in myoblast

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fusion (Yagami-Hiromasa *et al*., 1995), although other, as yet unknown, molecular partners are likely necessary to achieve plasma membrane merging (Yagami-Hiromasa *et al*., 1995).

Firm cell-to-cell adhesion is required before plasma membrane merging. Both the cysteine-rich and the disintegrin domains of ADAM12 provide molecular information for cell attachment (Iba *et al*., 1999, 2000; Zolkiewska, 1999; Thodeti *et al*., 2003). A model has been proposed in which initial low-affinity binding of the cysteine-rich domain by syndecan 4 triggers a conformational change of ADAM12-*cys*, allowing its binding to integrins (Iba *et al*., 2000). Both adhesion experiments to ADAM12 disintegrin domain by using cells expressing α_2, α_3, α_4, α_5, α_6, and α_9 integrins and cell-to-cell attachment between ADAM12 and α_9-expressing cells have shown that α_9 subunit is a preferred integrin partner for ADAM12, among integrins tested (Eto *et al*., 2000, 2002). The integrin α_9 subunit forms a single heterodimer $\alpha_9\beta_1$ (Palmer *et al*., 1993). Thus, this integrin constitutes a choice candidate as a privileged partner of ADAM12 during postnatal mpc fusion process (White, 2003).

Unlike $\alpha_4\beta_1$, an integrin closely related to $\alpha_9\beta_1$ and expressed by differentiating myogenic cells (Rosen *et al*., 1992), the role of $\alpha_9\beta_1$ in postnatal myogenesis has not been investigated (Mayer, 2003) despite previous mention of its expression at onset of myotube formation in the mouse embryo (Wang *et al*., 1995).

In the present article, we show that both ADAM12 and α_9 integrin are constitutively expressed during human myogenic cell differentiation and mediate a cell–cell interaction selectively involved in fusion of mononucleated myoblasts to preformed myotubes.

MATERIALS AND METHODS

Cell Culture

Unless indicated, culture media components were from Invitrogen (Paisley, Scotland) and culture plastics were from TPP AG (Trasadingen, Switzerland). Human mpc were cultured from muscle samples as described previously, in Ham's-F12 medium containing 15% fetal calf serum (FCS) (growth medium, GM) and antibiotics (Authier *et al*., 1999). Human mpc seeded at 5000 cells/cm^2 undergo proliferation and at day 4 of culture (nearly confluence), the medium was replaced with Ham's-F12 medium containing 5% FCS (differentiation medium, DM) to induce myogenic differentiation characterized by mpc fusion into myotubes (Baroffio *et al*., 1993). By using this procedure, mpc undergo standardized myogenic differentiation, including proliferation phase up to day 4, and then withdrawal of cell cycle to enter differentiation and formation of small myotubes (day 7), and growth of preformed myotubes through accretion of additional mpc (day 14). Only cultures presenting >95% cells expressing CD56/neural cell adhesion molecule (NCAM), a marker of myogenic cells (De Rossi *et al*., 2000) were used (clone 123C3, 1:20; Sanbio/Monosan, Uden, The Netherlands) (Chazaud *et al*., 2003).

mpc Growth and Differentiation

mpc growth curves are established by counting the cells with trypan blue dye exclusion (Authier *et al*., 1999). Myogenic differentiation is assessed by expression of myogenin as recommended previously (Fujio *et al*., 1999). Myogenin immunoblotting was carried out using 10 μg of mpc protein extract and M-225 antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA). mpc fusion was estimated by light microscopy examination of culture dishes after May-Grünwald Giemsa staining (Authier *et al*., 1999). Cultures were evaluated at the following stages: 1) proliferation stage (day 4 of culture, GM); 2) early differentiation stage (day 7 of culture, 3 d in DM); and 3) late differentiation stage (day 14 of culture, 10 d in DM). Experiments were run for each point at least on cultures from three individuals at passage 2.

The fusion index that helps assessment of ability of cells to fuse into multinucleated cells was calculated as the ratio of nuclei in myotubes to the total number of nuclei (Hirsch *et al*., 1998; Authier *et al*., 1999). To evaluate separately both formation of small nascent myotubes (2–4 nuclei) and growth of preformed myotubes, we performed nuclear number assays as described previously (Horsley *et al*., 2001). The number of nuclei in individual myotubes was counted for 50–200 myotubes. Myotubes were grouped in two categories: those with two to four nuclei and those with five or more nuclei. The percentage of myotubes in each category was calculated.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total mpc RNA (1 μg) prepared using the RNeasy mini kit (QIAGEN, Hilden, Germany) was reversely transcribed and amplified using OneStep RT-PCR kit (QIAGEN) and specific primers. Human ADAM12 is particular in that alternative splicing of the mRNA results in expression of two forms, a membrane-anchored form (ADAM12-L) and a shorter secreted form (ADAM12-S) (Gilpin *et al*., 1998). For ADAM12-S (GenBank accession no. AF23477), primers were 5′-GTT TGG CTT TGG AGG AAG CAC AG-3′ (sense) and 5′-GAA GCT CAA CCA GGA AGT CG-3′ (antisense). For ADAM12-L (GenBank accession no. AF23476), the sense primer was the same as for ADAM12-S and the antisense primer was 5′-GCA GCA ATC TCC TGG GAT TG-3′ (Gilpin *et al*., 1998). Amplifications were performed at 94, 60, and 72°C for 30, 45, and 45 s, respectively, for 40 cycles. For detection of α_9 and β_1 integrins mRNAs, we designed primers using Oligo 5.0 software. For α_9 integrin (GenBank accession no. NM_002207), the primers were 5′-GAT GAG TGG ATG GGG GTG AG-3′ (sense) and 5′-CAA TGG TGG ACG GGT GAG AG-3′ (antisense). For β_1 integrin (GenBank accession no. NM_033668), the primers were 5′-AGG ATT ACT TCG GAC TTC AGA-3′ (sense) and 5′-CTT TGG CAT TCA CAT TCA-3′ (antisense). Forty cycles of amplification were performed at 94, 60 (for α_9), or 55°C (for β1) and at 72°C for 30, 45, and 45 s, respectively. An endogenous human β2-microglobulin cDNA was amplified in parallel. Amplification products (10 μl) were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide (5 μg/ml) for visualization.

Electrophoresis and Immunoblotting

Cells were detached mechanically from culture flasks as described previously (Authier *et al*., 1999) and stored at −80°C in protease inhibitor cocktail for mammalian tissues (P8340; Sigma-Aldrich, St. Louis, MO). Cell pellet was incubated with lysis buffer [1% CHAPS (wt/vol), 150 mM NaCl, 50 mM Tris, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and gently shaken for 10 min on ice. The homogenate was centrifuged at 4000 × *g* for 10 min at 4°C. To detect secreted ADAM12-S, complete culture medium was replaced by Ham's-F12 without serum, and supernatants were collected after 24 h and concentrated using Vivaspin 15R concentrator (Vivascience, Hannover, Germany). Protein concentration was determined using bicinchoninic acid protein assay kit from Pierce Chemical (Rockford, IL). Aliquots corresponding to 20–50 μg of proteins were subjected to SDS-PAGE, followed by transfer to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Ponceau Red staining was used to ensure loading of equal amounts of proteins. All antibodies were diluted in phosphate-buffered saline (PBS)-0.1% Tween 20 (PBST). Staining of the blotted membranes was performed as follows: 1) blockade of the nonspecific binding by incubation with PBST-5% dry nonfat milk for 2 h at room temperature; 2) overnight incubation at 4°C with the primary antibody; 3) link-step either with a rabbit horseradish peroxidase-conjugated anti-rat Ig (1:1000; 1 h) in case of monoclonal primary antibody, or with goat horseradish peroxidase-conjugated anti-rabbit Ig (1:1000; 1 h) in case of polyclonal primary antibody. Revelation was performed by using the enhanced chemiluminescence kit (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom). Detection of ADAM12 was ensured by using a rat monoclonal antibody (mAb) (2F7) recognizing the disintegrin domain (Kawaguchi *et al*., 2002) and goat-polyclonal antibody directed toward cytoplasmic tail of ADAM12-L (sc-16527; Santa Cruz Biotechnology, Santa Cruz, CA) that of α_9 integrin by a rabbit anti-α_9 antiserum raised against cytoplasmic domain (#1057; generous gift from Dean Sheppard, University of California, San Francisco, San Francisco, CA) and that of myogenin by a rabbit polyclonal anti-myogenin (M-225; Santa Cruz Biotechnology). Controls ensured the lack of endogenous binding of the secondary antibody and the absence of peroxidase.

Immunofluorescence

For immunofluorescence procedure, mpc were cultured on glass coverslips and fixed in 1% paraformaldehyde. Primary antibodies included rabbit polyclonal antibodies to hADAM12 cysteine-rich domain (rb122) (Kawaguchi *et al*., 2002) (1/100; 37°C; 60 min), mouse mAb to $\alpha_9\beta_1$ integrin IgG1 (Y9A2; Chemicon International, Temecula, CA) (20 μg/ml; 4°C; overnight), and mouse mAb to myogenin (F5D; DakoCytomation Denmark A/S, Glostrup, Denmark) (1/20; 37°C; 60 min). Secondary antibody were fluorescein isothiocyanate (FITC)- or tetramethylrhodamine B isothiocyanate (TRITC)-conjugated secondary antibody (The Jackson ImmunoResearch Laboratories, Bar Harbor, ME; 1/250; 37°C; 60 min). For double staining, unpermeabilized mpc were first incubated with anti-$\alpha_9\beta_1$ antibody and then with anti-ADAM12 antibody. All antibodies were diluted in 2% FCS PBS. The specificity of the immunostaining was investigated by replacing primary antibodies with normal rabbit, goat, or mouse IgG fraction (10 or 20 μg/ml). Coverslips were mounted on slides with Vectashield medium containing 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (Vector Laboratories, Burlingame, CA). A Zeiss AXIOPHOT2 microscope was used for conventional fluores-

cence microscopy, and digital photographs were taken using Hammamatsu ORCA-ER camera.

Antisense Oligonucleotides

Antisense oligonucleotides and controls directed to ADAM12 were designed and manufactured by Biognostik (Göttingen, Germany). Sequences for ADAM12 antisenses were chosen to inhibit both isoforms: CTC TCT TTT ATG CCT TCT (position 909 in the prodomain) and CCC CAT TCC TTT CTC C (position 1512 in the disintegrin domain). Sequences were chosen because they were short enough to penetrate living cells and long enough to be sequence specific. Nucleotides in the 3′ and -5′ ends are modified oligonucleotides with phosphorothioate backbone modification conferring exonuclease resistance. Cross-examination of GenBank showed neither autohybridization between these oligonucleotides nor a complementary sequence in any other endogenous gene thus far entered into the database and especially with other ADAMs expressed in myogenic cells (ADAM9, ADAM17, and ADAM19). Random control oligos (nonsense in our legend) have been designed thoroughly to avoid toxic motifs and to be nonhomologous to any known sequence (ACT ACT ACA CTA GAC TAC and GCT CTA TGA CTC CCA G). In a first step, cellular uptake is monitored with FITC-labeled control oligonucleotides according to manufacturer's recommendations. In a second step, the translation of the targeted protein is inhibited specifically. ADAM12 antisense oligos were added at 2 μM to culture medium at time of switch in DM and are reapplied at the same concentration 2 d later in case of cultures evaluated at day 14. Inhibition of protein ADAM12 synthesis was quantified by immunoblotting on cell extracts for ADAM12-L and 24-h supernatants for ADAM12-S at day 14. Effect on fusion index was evaluated at day 5 and day 14 for each sample: untreated cells, antisense-treated cells, and nonsense-treated cells. For each experiment, 10 randomly chosen fields were evaluated for calculation of fusion index.

Blocking Antibodies

When GM was replaced with DM, the following mouse monoclonal blocking antibodies were added at saturating concentrations (10 μg/ml): anti-$\alpha_9\beta_1$ (clone Y9A2; Chemicon International), anti-VCAM-1/CD106 (clone 1G11), and anti-α_4/CD49d (clone HP2/1). Controls included cells grown in DM only and in DM with mouse isotype control (Vector Laboratories). Blocking antibodies were added to culture medium at time of switch in DM and are reapplied at the same concentration 2 d later in case of cultures evaluated at day 14. Effect on fusion index was evaluated at days 5 and 14: untreated cells, anti-$\alpha_9\beta_1$–treated cells, and mouse IgG-treated cells. For each experiment, 10 randomly chosen fields were evaluated for calculation of fusion index.

Coimmunoprecipitation Assay

To assess interaction between ADAM12 and $\alpha_9\beta_1$ during mpc fusion, we performed coimmunoprecipitation assays by using anti-ADAM12 and anti-$\alpha_9\beta_1$ antibodies prebound on protein G beads. Cells were scraped and homogenized in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, and 1 mM EGTA) containing protease inhibitors (P8340; Sigma-Aldrich). Protein extraction was done on ice by 10-min incubation with an equal volume of a modified radioimmunoprecipitation assay (RIPA) buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40, 10% glycerol, 10 mM EDTA, and protease inhibitors) and gently passaged through a 22-gauge needle. Cell extracts were centrifugated at 4°C to remove debris. Protein concentration was determined as described previously.

Coimmunoprecipitation was performed using protein G beads (Immunoprecipitation Starter Pack; Amersham Biosciences UK) because of mouse IgG1 affinity was higher for protein G than for protein A. Protein extracts (500 μg–1 mg) were precleared by incubation with protein G beads-Sepharose 4B (60 min at 4°C in an end-over-end mixer) followed by a centrifugation (10 min at 10,000 $\times$ *g*; 4°C). Equal volumes of cleared supernatants were used for different immunoprecipitations. Protein G beads were first prebound with anti-ADAM12 antibodies (6E6, 8F8, and 6C10 ascites mix; 10 μl), anti-$\alpha_9\beta_1$ antibody (Y9A2; 20 μg), or irrelevant mouse Ig (20 μg) (60 min at 4°C in an end-over-end mixer), and then immunoprecipitation was conducted overnight at 4°C in an end-over-end mixer. Immunocomplexes on beads were centrifuged (250 $\times$ *g*; 2 min), washed twice with RIPA buffer; and resuspended in an appropriate volume of 2$\times$ SDS sample buffer (1% SDS, 100 mM dithiothreitol, and 50 mM Tris, pH 7.5) and boiled (10 min) to detach precipitated proteins. After centrifugation at 17,000 $\times$ *g*, samples were subjected to gel migration in denaturing conditions (SDS-PAGE), transfer on nitrocellulose membrane, and after rouge ponceau staining, immunoblotting with antibodies different from those used for immunoprecipitation: rabbit polyclonal anti-α_9 antibody (#1057) and rabbit polyclonal anti-ADAM12 (rb122).

Cell Adhesion Assays

A first set of experiments was designed to evaluate the ability of $\alpha_9\beta_1$ expressed at mpc surface to bind ADAM12. Ninety-six well plates were first incubated overnight at 4°C with recombinant full-length human ADAM12 (Loechel *et al*., 2000). Recombinant full-length human ADAM12 (10 μg/ml in coating buffer [6.22 g/l $NaHCO_3$, 1.70 g/l Na_2CO_3, and H_2O, pH 9.5]) and then saturated with 1% bovine serum albumin in PBS (37°C; 60 min). mpc were seeded (0.6 $\times$ 10^6 cells/ml) and allowed to attach 60 min at 37°C in presence of Y9A2, mAb to $\alpha_9\beta_1$ (20 μg/ml in Ham's-F12) or O26, monoclonal blocking antibody to α_7 (20 μg/ml; generous gift from S. J. Kaufman, University of Illinois, Urbana, IL) (Crawley *et al*., 1997). Controls were ensured by replacement of Y9A2 and O26 by normal IgG fraction (20 μg/ml in Ham's-F12). mpc adhesion was evaluated using crystal violet staining (Chazaud *et al*., 2002).

A second set of experiments was designed to assess involvement of different molecules in natural adhesion to their support of cultured mpc. Mpcs were seeded in 96-well plates (10,000/cm^2 in GM) and allowed to attach overnight. At subconfluence, GM was replaced by DM supplemented with different effectors (10 μg/ml), including RGD peptide (600 μg/ml, G5646; Sigma-Aldrich) and monoclonal blocking antibodies to $\alpha_9\beta_1$ (Friday *et al*., 2003), α_4 (Sanchez-Madrid *et al*., 1986), or VCAM-1 (Thornhill *et al*., 1991). mpc deadhesion was evaluated after 4 h and 3 d by using crystal violet staining (Chazaud *et al*., 2002).

A third set of experiments was designed to evaluate the ability of ADAM12, $\alpha_9\beta_1$, and $\alpha_7\beta_1$ expressed at mpc surface to mediate cell-cell adhesion. Cultured cells were separated in two parts. A part of cells was seeded at 50,000 cells/cm^2 in 96-wells plates and treated or not with antisense or nonsense (2 μM) during 72 h and allowed to differentiate in small myotubes. During the same time, the second part of cells was seeded at 5000 cells/cm^2 in 75-cm^2 flasks with 5-bromo-2-deoxyuridine (BrdU) (1 μM, referenced as mpc-BrdU) and with antisense or nonsense (2 μM; referenced, respectively, as mpc-AS-BrdU and mpc-NS-BrdU). At day of assay, these cells were detached from culture flasks by a nonenzymatic solution to preserve membrane adhesion molecules (cell dissociation solution; Sigma-Aldrich Chemie, Steinheim, Germany), incubated as described previously with blocking antibodies (Y9A2, O26, or IgG fraction), and allowed to attach 90 min on cell layer in 96-wells plates. Adherent cells were detected by the measurement of BrdU incorporation during DNA synthesis by using the cell proliferation enzyme-linked immunosorbent assay, BrdU (colorimetric) (Roche Diagnostics, Manheim, Germany). Optical density was read at 450 nm.

Statistics

All experiments were performed using at least three different cultures. Statistical analyses were achieved with paired *t* test and Kruskal–Wallis test, by using InStat 3.0 (GraphPad Software, San Diego, CA). A p value <0.05 was considered significant.

RESULTS

Human mpc Fusion

Human mpc were cultured in GM until subconfluence, and at day 4 DM was used to boost myogenesis. mpc density and differentiation were evaluated at the stage of proliferation (day 4), early differentiation (day 7), and late differentiation (days 14 and 21). Myogenesis was assessed by both fusion index and myogenin expression. Shift from GM to DM allows to increase fusion index, reaching 66 $\pm$ 0.43% at day 14 and finally 67 $\pm$ 2% at day 21 (Figure 1A), whereas spontaneously it does not exceed an average of 30%. As assessed by myogenin expression, mpc truly undergo differentiation program in these conditions (Figure 1B). As assessed by plateauing of both cell density from day 7 to day 14 and fusion index from day 14 to day 21, the increase of fusion index observed in this time lapse (7–14) likely reflected elongation of existing myotubes rather than appearance of newly formed myotubes (Figure 1C).

Human mpc Constitutively Express ADAM12 during In Vitro Myogenesis

RT-PCR showed expression of both short (S) and long (L) isoforms of ADAM12 by human mpc, at the three stages of differentiation (Figure 2A). Immunoblots assessed the production of both ADAM12-L and -S proteins at the three stages of differentiation (Figure 2B). In mpc extracts, ADAM12-L was revealed as two immunoreactive bands corresponding to ADAM12-L proform (110 kDa) and mature ADAM12-L (90 kDa) (Figure 2B). In mpc supernatants, ADAM12-S was detected as ADAM12-S proform (92 kDa) and mature ADAM12-S (68 kDa) (Figure 2B). Cell-associated ADAM12-L expression and release of ADAM12-S from mpc

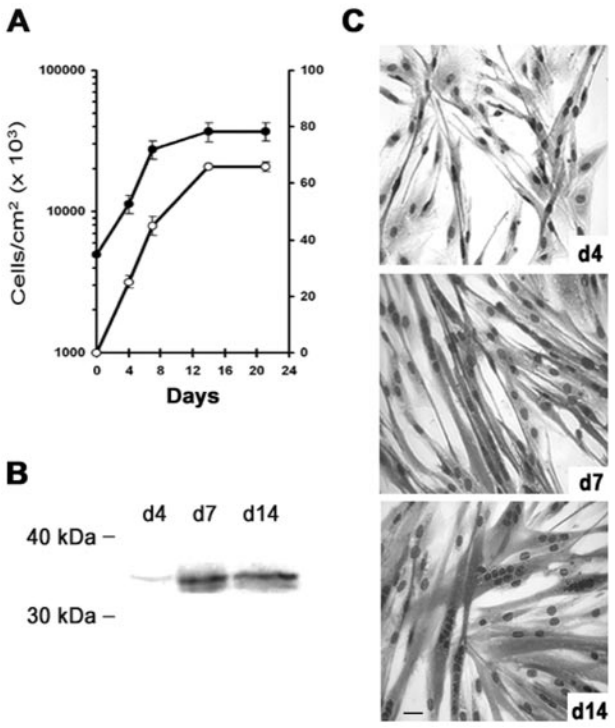

Figure 1. In vitro differentiation of human mpc. (A) mpc growth is expressed in number of cells per square centimeter (closed circles), and mpc differentiation is estimated by the fusion index (open circles). There is no significant increase of fusion index from day 14 to day 21. Results are means ± SD of eight independent cultures. (B) Myogenin immunoblot at days 4, 7, and 14 of mpc culture. (C) May-Grünwald Giemsa stain of mpc at day 4 (d4), 7 (d7), and 14 (d14) of culture. Bar, 40 μm.

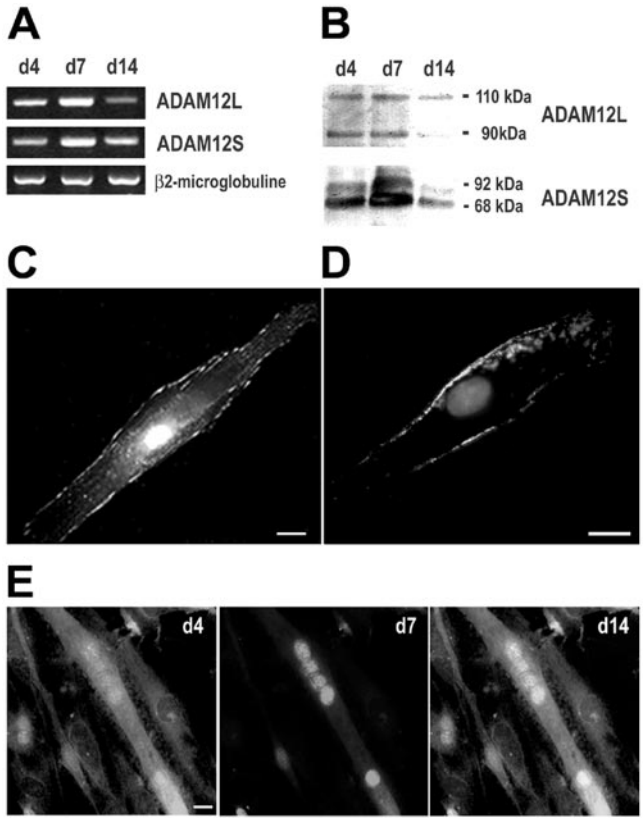

Figure 2. ADAM12 expression by human mpc. (A) RT-PCR analysis of ADAM12-L, ADAM12-S, and β2-microglobulin mRNAs in mpc at days 4, 7, and 14 of culture (expected sizes of PCR products were 314, 371, and 335 base pairs, respectively). (B) Immunoblot analysis of pro- (100-kDa) and mature (90-kDa) ADAM12-L (top) in mpc lysates with goat polyclonal anti-cytoplasmic tail of ADAM12-L, and of pro- (92-kDa) and mature (68-kDa) ADAM12-S (bottom) in mpc supernatants with mouse monoclonal 2F7 directed toward disintegrin domain of ADAM12, at day 4, 7, and 14. (C) ADAM12 localization (FITC) in Triton X-100 permeabilized mpc by using rb122 antibody. (D) ADAM12 localization (FITC) in unpermeabilized mpc. (E) After permeabilization, differentiated mpc (day 7) were labeled with rb122 antibody revealed by FITC-conjugated secondary antibodies (green) and with myogenin antibody revealed by TRITC-conjugated secondary antibodies (red). Nuclei staining with DAPI. Bars, 20 μm.

is similar to that observed in several other cell types expressing ADAM12 (Gilpin *et al*., 1998; Cao *et al*., 2002). Note that a fraction of the ADAM12-S proform does not get processed by human mpc and is secreted in culture medium as shown previously (Loechel *et al*., 1998). Immunostaining of ADAM12 in mpc after plasma membrane permeabilization revealed both intracellular and plasma membrane localization of ADAM12 (Figure 2C). Such a distribution in the murine myogenic cell line C2C12 (Cao *et al*., 2002) and other cell types (Hougaard *et al*., 2000; Kadota *et al*., 2000) has been shown to reflect association to the organelles composing the secretory pathway, i.e., endoplasmic reticulum, Golgi apparatus, and post-Golgi components. Moreover, plasma membrane labeling showed as discrete and elongated spots distributed throughout the cell body and plasma membrane projection as lamellipodiae (Hougaard *et al*., 2000; Kadota *et al*., 2000) (Figure 2C). ADAM12 immunostaining on unpermeabilized cell showed extensive membrane labeling (Figure 2D). Coexpression of the myogenic transcription factor myogenin and ADAM12 in single myotubes confirmed that differentiating myogenic cells also express ADAM12 (Figure 2E).

$\alpha_9\beta_1$ Integrin Is Also Expressed during mpc Differentiation

It is well established that β_1 integrin subunit is expressed by both myoblasts and myotubes (Dickson *et al*., 1992; Hirsch *et al*., 1998); and consistently, β_1 mRNAs were detected by RT-PCR (Figure 3A). RT-PCR also showed expression of α_9 integrin subunit mRNAs at the three stages of myogenesis (Figure 3A). Immunoblots performed with antiserum #1057 assessed the production of α_9 integrin protein at the same time points in the form of immunoreactive band corresponding to the mature α_9 (150 kDa) (Figure 3B). Immunofluorescence on unpermeabilized cells confirmed expression of $\alpha_9\beta_1$ integrin at the membrane throughout in vitro myogenesis (Figure 3C). Double immunostaining showed that coexpression of both ADAM12 and $\alpha_9\beta_1$ was mainly observed when cells have entered differentiation. Colocalization of ADAM12 and $\alpha_9\beta_1$ could be observed at membrane cell surface and at close cell-cell contact (Figure 4).

$\alpha_9\beta_1$ Integrin Acts as a Ligand for mpc Membrane-bound ADAM12

Coimmunoprecipitation experiments using mpc extracts incubated with antibodies to ADAM12 and subjected to immunoblotting with anti-α_9 antibody disclosed an expected immunoreactive band at 150 kDa, corresponding to α_9, thus confirming binding of α_9 to ADAM12. Inverse experiments

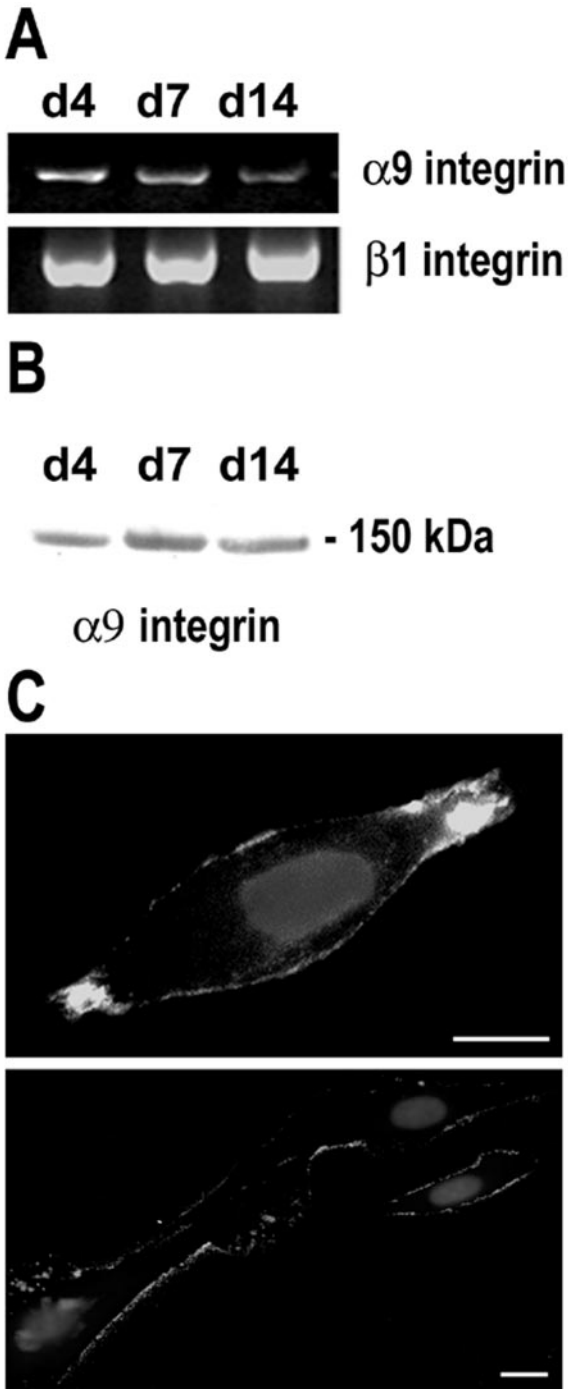

Figure 3. $\alpha 9\beta 1$ expression by human mpc. (A) α_9 and b_1 integrin subunits in mpc at days 4, 7, and 14 of culture, analyzed by RT-PCR. Expected size of PCR products was, respectively, 424 and 881 base pairs. (B) Detection by immunoblot analysis of mature α_9 (150 kDa) in cultured cell lysate at days 4, 7, and 14. (C) Unpermeabilized mpc exhibited membrane immunolabeling of $\alpha_9\beta_1$ integrin with monoclonal Y9A2 antibody (directed toward $\alpha_9\beta_1$) by using FITC-conjugated secondary antibody. Propidium iodide stain of nuclei. Bars, 20 μm.

using anti-$\alpha_9\beta_1$ antibody followed by immunoblotting with anti-ADAM12 antibody disclosed an immunoreactive band at 90 kDa, corresponding to mature membrane-bound ADAM12 (Figure 6A).

Inhibition of ADAM12/$\alpha_9\beta_1$ Integrin Interplay Inhibits mpc Fusion

To investigate the role of ADAM12–$\alpha_9\beta_1$ integrin interplay in human mpc differentiation, we incubated mpc cultures with ADAM12 antisense oligonucleotides or $\alpha_9\beta_1$ blocking antibodies at time of differentiation induction. At day 14, cells cultured in the presence of ADAM12 antisense oligonucleotides showed dramatic expression drop of both ADAM12-S and -L isoforms as assessed by immunoblotting (inhibition by 91 ± 8.5%, $p < 0.001$ and 85 ± 1.5%, $p < 0.001$, respectively) (Figure 5A). When subjected to ADAM12 antisense oligonucleotides or $\alpha_9\beta_1$ blocking antibody, morphology of cultures changed, with myotubes being less numerous and showing a thinner diameter, and a lower number of nuclei than controls, despite a subnormal length (Figure 5B). When incubated with either nonsense oligonucleotides or isotypic immunoglobulins, cultures displayed normal morphology with similar myotube size and nuclei content (Figure 5B).

Significant inhibition of fusion was assessed by fusion index decrease at day 14 (inhibition by 46.7 ± 4.3% at day 14, $p < 0.01$) compared with untreated cells (Figure 5C). Similarly, $\alpha_9\beta_1$ blocking antibodies used at saturating concentrations (Eto *et al.*, 2000) induced fusion index decrease by 48.2 ± 1% at day 14 ($p < 0.01$). When both ADAM12 antisense oligonucleotides and $\alpha_9\beta_1$ blocking antibodies were used, a further decrease of mpc fusion was observed: 62.2 ± 2.4% at day 14 ($p < 0.001$) (Figure 5C).

ADAM12 and $\alpha_9\beta_1$ Are Mainly Operative in Myotube Growth

To assess the role of ADAM12 and $\alpha_9\beta_1$ integrin in formation and growth of myotubes that represent two distinct phases of myogenesis, we analyzed the effects of ADAM12 antisense and anti-$\alpha_9\beta_1$ antibody with regard to the size of myotubes. These effectors were added at time of shift (day 4) and 2 d later (day 6). Evaluation of early differentiation was done at day 5, when first small nascent myotubes (no cell with more than 4 nuclei are formed. Both effectors induced a decrease of nuclei included in small myotubes (2–4 nuclei) by 18% ($p < 0.0001$), suggesting delayed early differentiation.

Evaluation at day 14 (late differentiation) showed an increased proportion of mononucleated cells and a twofold decrease of the proportion of nuclei included in large myotubes (≥5 nuclei), whereas a proportion of nuclei included in small myotubes (containing 2–4 nuclei) was similar to controls (Figure 5D). Then, we used a previously described nuclear number assay, in which the number of nuclei inside myotubes according to their size is established. Myotubes were separated into two groups: small myotubes with two to four nuclei and large myotubes with five or more nuclei. Then, we calculated the percentage of nuclei belonging to each group. This assay showed that the number of nuclei incorporated into small myotubes was significantly higher and that of nuclei incorporated into large myotubes significantly lower than in controls after exposure of cultures to ADAM12 antisense oligonucleotides and/or anti-$\alpha_9\beta_1$ antibody (Figure 5E). This result indicates that both effectors inhibit growth of myotubes (by accretion of additional nuclei to small preformed myotubes) more than formation of nascent myotubes.

ADAM12–$\alpha_9\beta_1$ Constitutes a Potent Adhesion System

To further explore significance of ADAM12–$\alpha_9\beta_1$ interaction, we seeded mpc on full-length ADAM12-coated plates.

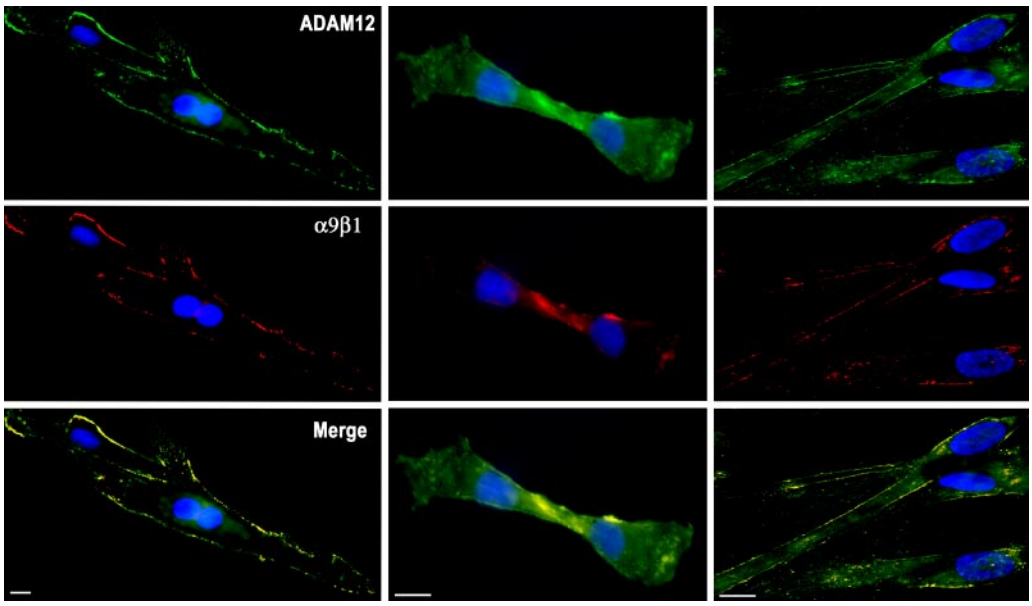

Figure 4. Membrane expression of ADAM12 and $\alpha_9\beta_1$ integrin and colocalization at cell-cell contact. Double immunolabeling on unpermeabilized mpc of ADAM12 (rb122) and $\alpha_9\beta_1$ integrin (Y9A2) by using TRITC- or FITC-conjugated secondary antibodies, respectively. DAPI stain of nuclei. Bar, 20 μm.

Mpc were adherent on this support and anti-$\alpha_9\beta_1$ blocking antibody (Y9A2) induced an inhibition of mpc adherence to ADAM12-coated plates by 60.4 ± 10.6% ($p < 0.05$) (Figure 6B). Because the disintegrin domain of ADAM12 was shown to bind to $\alpha_7\beta_1$ integrin (Zhao *et al*., 2004), we compared the respective inhibitory effects of both Y9A2 and anti-α_7 blocking antibody (O26) on mpc adhesion on ADAM12-coated plates. In the same conditions, blockade of α_7 integrin inhibited mpc adhesion at lower level (21 ± 3.6%) than Y9A2 ($p < 0.05$) (Figure 6B). Together, these results strongly support actual $\alpha_9\beta_1$ integrin binding to full-length ADAM12, the membrane-bound isoform of ADAM12.

$\alpha_9\beta_1$ Integrin Binding to ADAM12 Induces Intercellular mpc Adhesion

Because both ADAM12 and $\alpha_9\beta_1$ integrin may induce cell-to-cell adhesion (Eto *et al*., 2000), we also evaluated whether inhibition of fusion could be related to a nonspecific alteration of mpc attachment to their self-produced extracellular matrix. Detachment of mpc was assessed under the treatments used in fusion inhibition assays. Antibodies to either VLA-4 or VCAM-1 induced significant mpc detachment after 4 h or 3 d ($p < 0.03$ for each molecule at each time point) (Figure 7A). By contrast, no mpc detachment was observed after exposure to either $\alpha_9\beta_1$ blocking antibody (at 4 h and 3 d) or ADAM12 antisense oligonucleotides (3 d) (Figure 7A). Accordingly, treated and untreated cell cultures looked similar. These results indicate that ADAM12 and $\alpha_9\beta_1$ interact predominantly at the intercellular level.

The role of ADAM12 and $\alpha_9\beta_1$ in prefusional mpc adhesion was evaluated using adhesion assays on a cell layer. Treatment of mpc-BrdU by ADAM12 antisense oligonucleotides (mpc-AS-BrdU) or incubation of mpc-BrdU with anti-$\alpha_9\beta_1$ (Y9A2) inhibited their adhesion on untreated differentiating mpc by 51.6 ± 1.3% ($p < 0.05$) and 52.4 ± 7% ($p < 0.05$) respectively (Figure 7, B and C). Combination of both inhibition strategies (mpc-AS-BrdU + Y9A2) further decreased mpc adhesion by 65.7 ± 2.5% ($p < 0.01$) (Figure 7D). Interestingly, the observed inhibition level of mpc-mpc adhesion was comparable with that of mpc fusion. Because $\alpha_7\beta_1$ integrin may interact with disintegrin and cysteine-rich domains of ADAM12 to mediate cell adhesion (Zhao *et al*., 2004), we evaluated its implication in our model. The anti-α_7 antibody O26 did not induce significant inhibition of mpc adhesion regardless of what mpc-BrdU was used: antisense, nonsense, or controls (Figure 7). In a second set of experiments, we replaced the untreated differentiating mpc by a layer of mpc previously subjected to ADAM12 antisense oligonucleotides. When mpc-BrdU was put on to adhere, inhibition of adhesion was increased: 57.4 ± 2.6, 61.2 ± 1.3, and 70.1 ± 0.2% for mpc-BrdU + Y9A2, mpc-AS-BrdU, and mpc-As-BrdU + Y9A2, respectively (Figure 7). As expected, O26 did not induce any significant effect (Figure 7, B–D). Together, our results confirmed that ADAM12 binding to $\alpha_9\beta_1$ mediates mpc adhesion to differentiating mpc. Moreover, interaction of ADAM12 and $\alpha_9\beta_1$ in mpc adhesion to nascent myotubes seems to be bidirectional, as suggested by expression analyses showing that mpc similarly express ADAM12 and $\alpha_9\beta_1$ during their differentiation.

DISCUSSION

In the present study, we show that ADAM12 and $\alpha_9\beta_1$ integrin are coexpressed during in vitro differentiation of human myogenic cell, that ADAM12 and α_9 coimmunoprecipitate and are instrumental in intercellular mpc adhesion, and that both molecules participate to mpc fusion to myotubes.

ADAM12 is a multidomain protein that could promote myogenic differentiation by exerting several different activities at different times, either individually or through multimolecular interactions (Engvall and Wewer, 2003). For example, ADAM12 cell signaling may positively influence myogenesis, because membrane-bound ADAM12 activates phosphatidylinositol (PI) 3-kinase by mediating its recruitment to the membrane (Kang *et al*., 2001), active PI 3-kinase

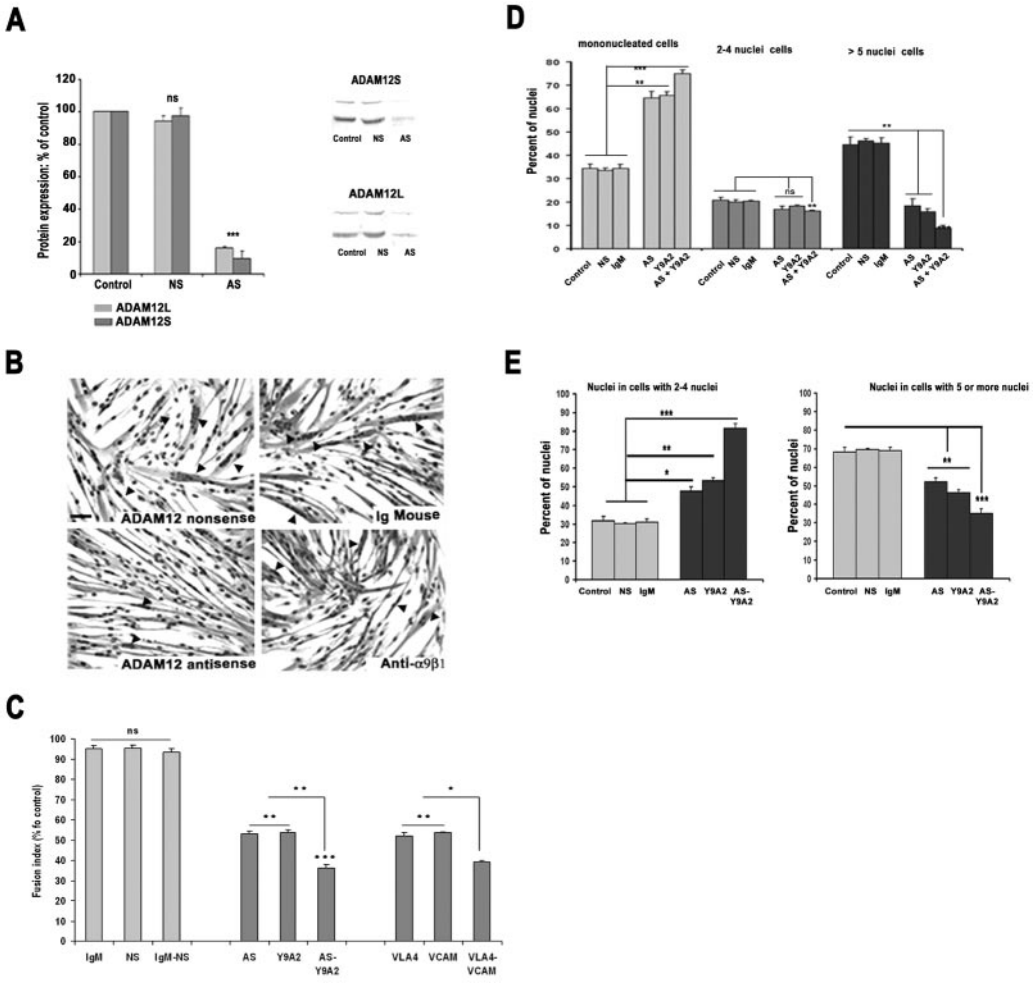

Figure 5. ADAM12 and $\alpha_9\beta_1$ integrin in mpc fusion. (A) Immunoblot quantification of ADAM12 isoforms expression by mpc-treated or not with an ADAM12 antisense and nonsense. Graph shows densitometric analysis of three different cultures, and immunoblot presents an example of one culture. (B) May-Grünwald Giemsa stain of mpc at day 14 showing morphological appearance of myotubes after different treatments. Bar, 40 μm. (C) Fusion index was measured for each situation and normalized as percentage of the control (untreated cells). Results are means ± SD of three experiments run in duplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (D) Percentage of nuclei included in mononucleated cells, in cells with two to four nuclei and in cells with five or more nuclei under the total number of nuclei (**$p < 0.01$, ***$p < 0.001$). (E) Percentage of nuclei included in myotubes with two to four nuclei (left) or five or more nuclei (right) under the total number of nuclei in myotubes with two or more nuclei (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

being crucially involved in terminal differentiation of myogenic cells (Jiang *et al*., 1998; Li *et al*., 2000). Moreover, the ADAM12 cytoplasmic tail binds α-actinin 1 and 2 (Galliano *et al*., 2000; Cao *et al*., 2001), this binding being required for full-blown fusion of C2C12 myogenic cells (Galliano *et al*., 2000).

ADAM12 also may exert protease activity, leading to growth factor release (Asakura *et al*., 2002). The secreted form ADAM12-S can cleave insulin-like growth factor (IGF)-binding protein-3 and -5 (Loechel *et al*., 2000; Shi *et al*., 2000), and in so doing promotes bioactivity of IGF-1 and -2, two positive regulators of muscle growth, survival, and regeneration (Husmann *et al*., 1996; Kaliman *et al*., 1996; Kronqvist *et al*., 2002).

In addition, ADAM12 may influence integrin-mediated cell adhesion functions. In mouse, ADAM12 positively modulates expression of integrin α_7 (Cao *et al*., 2003; Moghadaszadeh *et al*., 2003), which has functions in skeletal muscle development and disease (Burkin and Kaufman, 1999). $\alpha_7\beta_1$ integrin is mainly involved in mpc adhesion and migration on the matrix component laminin (Yao *et al*., 1996; Crawley *et al*., 1997; Blanco-Bose and Blau, 2001) and in myotendinous junction formation (Nawrotzki *et al*., 2003). Murine $\alpha_7\beta_1$ integrin can bind ADAM12 disintegrin and cysteine-rich domain and induce cell adhesion (Zhao *et al*., 2004). In our model however, ADAM12–α_7 interaction was not crucially involved in cell-cell adhesion as assessed by α_7 blockade experiments.

Our data indicate that ADAM12 binding to $\alpha_9\beta_1$ is involved in mpc fusion. mpc fusion results from an ordered sequence of events, including cell migration, alignment, recognition, adhesion, and membrane merging. Any effector hindering one of these steps decreases the fusion rate. In our model, differentiation was induced when mpc were at subconfluence state to minimize the role of cellular motility in myotube formation. mpc adhesion to extracellular matrix also was considered, because both ADAM12 and $\alpha_9\beta_1$ integrin may bind directly or indirectly extracellular matrix components such as fibronectin (Kawaguchi *et al*., 2003) or tenascin-C (Marcinkiewicz *et al*., 2000). In our experiments,

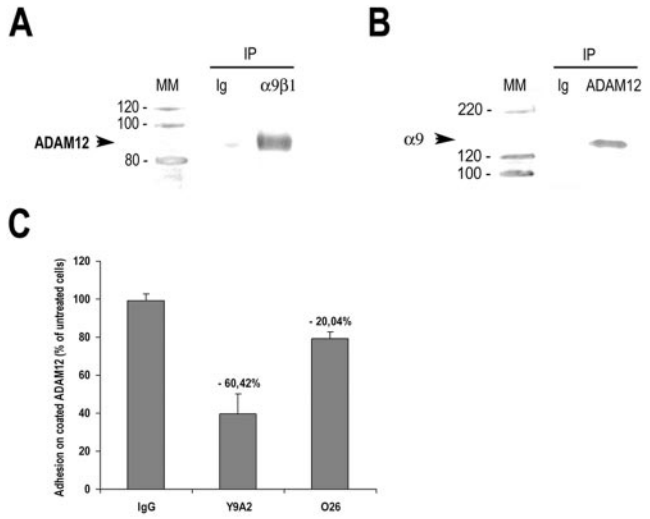

Figure 6. $\alpha_9\beta_1$ integrin acts as a ligand for mpc membrane-bound ADAM12. (A) Immunoblot analysis of ADAM12 expression (with rb122, anti-disintegrin domain) after coimmunoprecipitation with irrelevant mouse immunoglobulins or monoclonal anti-Y9A2 antibody (left), and of α_9 expression (with #1057, anti-cytoplasmic domain) after coimmunoprecipitation with irrelevant mouse immunoglobulins or monoclonal anti-ADAM12 antibodies (6E6, 8F8, and 6C10 mix) (right). Example of one blot. (B) Percentage of bound cells to plate coated with purified full length ADAM12 and incubated with either irrelevant mouse Ig, anti-$\alpha_9\beta_1$ (Y9A2), or anti-a_7b_1 (O26) blocking antibodies. Results are means ± SD of three experiments run in triplicate ($p < 0.05$).

inhibition of ADAM12–$\alpha_9\beta_1$ interactions induced marked decrease of mpc fusion, without altering their adhesion to their own matrix. This contrasted with inhibition of VCAM1–$\alpha_4\beta_1$ integrin interactions that decreased both mpc adhesion to support and fusion.

The key role of β_1 integrins in mpc fusion was recently stressed by using β_1-deficient transgenic murine cell lines (Schwander *et al*., 2003). In vitro, β_1-deficient mpc can establish cell-to-cell adhesion, but they are unable to proceed into myotube formation (Schwander *et al*., 2003). β_1 integrins may form a complex with the tetraspanin CD9 (Hemler, 2001), a molecule involved in murine mpc fusion and myotube maintenance (Tachibana and Hemler, 1999), and, in this way, play a part in mpc fusion (Schwander *et al*., 2003). However, fusion defects are rescued when β_1-deficient and wild-type mpc are mixed, indicating that β_1 integrins have to establish heterophilic interactions with another cell surface receptor, which may belong to ADAMs (Hirsch *et al*., 1998; Schwander *et al*., 2003). The concept that ADAMs may launch plasma membrane merging originated from the implication of the sperm surface protein fertilin in gamete fusion (Primakoff and Myles, 2002). Of note, however, none of ADAMs currently known to be expressed on sperm surface has been shown to directly act as a sperm-egg membrane merger (He *et al*., 2003). Therefore, it seems likely that ADAM12 and $\alpha_9\beta_1$ integrin are primarily involved in cell-cell adhesion processes.

In mammals, most molecules known to participate to muscle cell fusion were simply regarded as mediating myotube formation (Rosen *et al*., 1992; Yagami-Hiromasa *et al*., 1995; Barnoy *et al*., 1996; Gorza and Vitadello, 2000). Evidence emerges, however, that formation of small nascent myotubes is distinct from the subsequent accretion of mpc to performed myotubes, as initially shown in *Drosophila* (Rau *et*

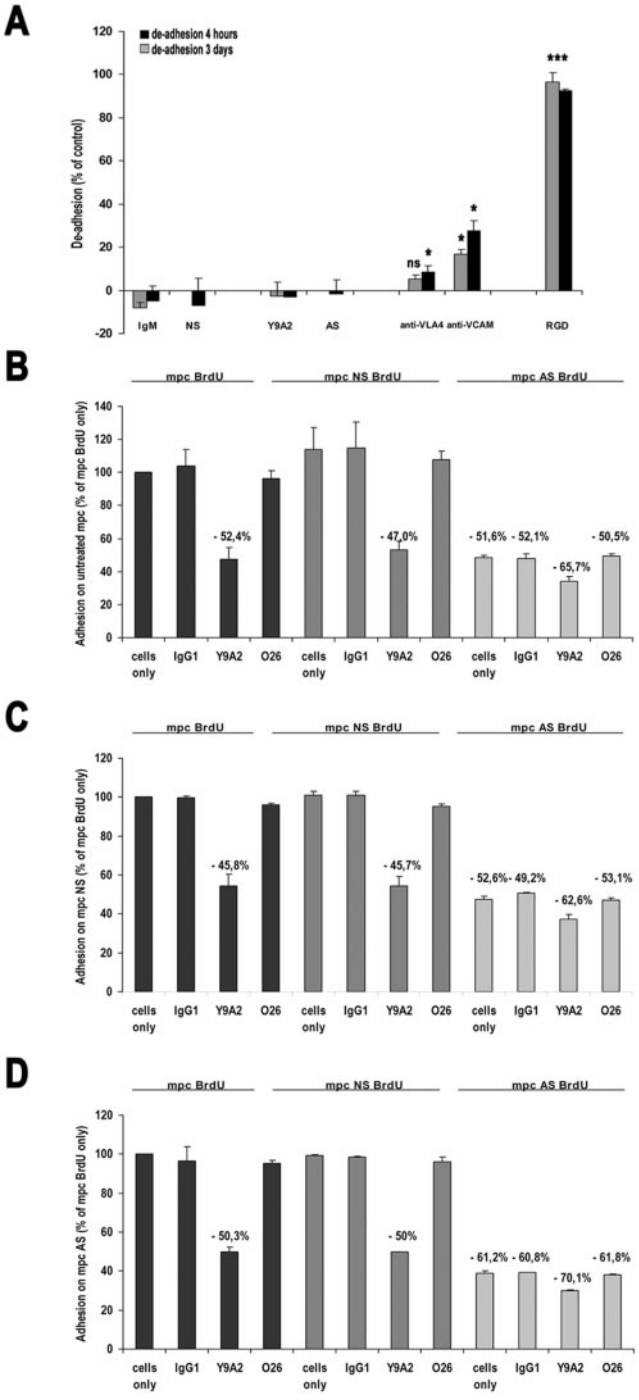

Figure 7. $\alpha_9\beta_1$ integrin binding to ADAM12 induces intercellular mpc adhesion. (A) Cell deadhesion was measured for each situation and normalized as percentage of the control (untreated cells). Results are means ± SD of three experiments run in triplicate (*$p < 0.03$). (B) Adhesion of cells treated or not with nonsense or antisense oligonucleotides on untreated cell layer after incubation with mouse immunoglobulins, anti-$\alpha_9\beta_1$ (Y9A2), or anti-a_7b_1 (O26) blocking antibodies. (C) Adhesion of cells treated or not with nonsense or antisense oligonucleotides on nonsense treated cell layer after incubation with mouse immunoglobulins, anti-$\alpha_9\beta_1$ (Y9A2), or anti-$\alpha_7\beta_1$ (O26) blocking antibodies. (D) Adhesion of cells treated or not with nonsense or antisense oligonucleotides on antisense treated cell layer after incubation with mouse immunoglobulins, anti-$\alpha_9\beta_1$ (Y9A2), or anti-a_7b_1 (O26) blocking antibodies. Results are means ± SD of three experiments run in triplicate.

al., 2001) and confirmed in mammals (Horsley *et al*., 2001; Horsley and Pavlath, 2003). Our results suggest that ADAM12 and its ligand $\alpha_9\beta_1$ integrin constitute a molecular system that is predominantly, but not exclusively, involved in myotube elongation. Molecular mechanisms that regulate mpc fusion with preformed myotubes in mammals include activation of the nuclear factor of activated T cells (NFAT)c2 pathway and interleukin (IL)-4/IL–4Rα interactions (Horsley *et al*., 2003; Pavlath and Horsley, 2003). Interestingly, the ADAM12 signaling pathway includes activation of PI3-kinase, a molecule involved in expression of both NFAT (Jascur *et al*., 1997) and IL-4 (Hirasawa *et al*., 2000). It could be appropriate to further investigate relationships of the different systems at work in myotube elongation and growth.

Cell therapy using transplantation of exogenous mpc aimed at fusing with mature muscle fibers may help treating devastating muscle diseases. Unfortunately, inability of mpc to fuse efficiently with host myofibers represents a major limitation of cell therapy in this setting (Skuk and Tremblay, 2000). Further understanding of the role of ADAM12 and $\alpha_9\beta_1$ integrin in mpc fusion is needed with expectance that manipulation of the system aimed at increasing mpc fusion to preexisting muscle cells could improve future therapeutic strategies.

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