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Melanoma cell adhesion molecule is a novel marker for human fetal myogenic cells and affects myoblast fusion

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Summary

Myoblast fusion is a highly regulated process that is important during muscle development and myofiber repair and is also likely to play a key role in the incorporation of donor cells in myofibers for cell-based therapy. Although several proteins involved in muscle cell fusion in *Drosophila* are known, less information is available on the regulation of this process in vertebrates, including humans. To identify proteins that are regulated during fusion of human myoblasts, microarray studies were performed on samples obtained from human fetal skeletal muscle of seven individuals. Primary muscle cells were isolated, expanded, induced to fuse in vitro, and gene expression comparisons were performed between myoblasts and early or late myotubes. Among the regulated genes, melanoma cell adhesion molecule (M-CAM) was found to be significantly downregulated during human fetal muscle cell fusion. M-CAM expression was confirmed on activated myoblasts, both in vitro and in vivo, and on myoendothelial cells (M-CAM$^+$ CD31$^+$), which were positive for the myogenic markers desmin and MyoD. Lastly, in vitro functional studies using M-CAM RNA knockdown demonstrated that inhibition of M-CAM expression enhances myoblast fusion. These studies identify M-CAM as a novel marker for myogenic progenitors in human fetal muscle and confirm that downregulation of this protein promotes myoblast fusion.

Keywords

Melanoma cell adhesion molecule (M-CAM); Myoblast; Cell fusion; Human skeletal muscle

Introduction

During embryonic development, multinucleated skeletal muscle fibers arise from the fusion of mononuclear myogenic cells that migrate from the somites and form primary myofibers (Buckingham, 2001; Parker et al., 2003; Gros et al., 2005; Relaix et al., 2005). These subsequently mature and grow in size by fusion of additional myogenic cells. These processes are thought to be highly regulated, involving numerous changes in gene expression, including genes encoding cell surface proteins mediating cell-to-cell recognition, adhesion and ultimately fusion (Chen and Olson, 2004; Chen and Olson, 2005). Differentiation of skeletal myoblasts into multinucleated myotubes can be recapitulated in vitro. Individual resident myoblasts can be enzymatically dissociated from primary tissue, expanded in vitro and induced to differentiate into multinucleated myotubes (Bischoff and Holtzer, 1969; Richler and Yaffe,

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1970; Hartley and Yablonka-Reuveni, 1990; Rando and Blau, 1994). Identification of critical molecules that mediate muscle cell adhesion, recognition and fusion is important not only to gain insight into the basic processes of muscle cell differentiation, but also to understand how mononuclear myogenic cells can be induced to incorporate into myofibers efficiently in cell-based therapy for muscle diseases. Thus, identification of key proteins regulated during human myoblast fusion can be useful from a therapeutic perspective.

Cell adhesion molecules are a broad family of proteins expressed in various cell types. They regulate cell-cell as well as cell-matrix interactions in normal tissues and have been shown to have a role in tumor tissue invasion and metastasis formation. One such molecule is the melanoma cell adhesion molecule (M-CAM), also known as CD146, Mel-CAM, MUC18, A32 antigen and S-Endo-1 (Shih, 1999). M-CAM is a membrane glycoprotein within the immunoglobulin superfamily containing the characteristic V-V-C2-C2-C2 domain structure. The protein has been identified in a multitude of 'normal' tissues, including several neuronal cell types (Hampel et al., 1997; Taira et al., 2004; Taira et al., 2005), endothelial cells (Anfosso et al., 1998; Bardin et al., 2003) and smooth muscle (Shih et al., 1998). Multiple roles are suggested for M-CAM in various tissues including implantation and placentation (Yoshioka et al., 2003; Liu et al., 2004), the rearrangement of actin in the cytoskeleton (Anfosso et al., 2001) as well as a role in cohesion of the endothelial monolayer (Anfosso et al., 1998; Anfosso et al., 2001). M-CAM has also been identified in several neoplasms including melanoma, angiosarcoma and hemangioma (Li et al., 2003). However, the data describing the role of M-CAM in tumors is conflicting. This protein has been shown to increase expression in melanoma during tumor progression into metastasis (Nyormoi and Bar-Eli, 2003), whereas other studies demonstrated that the transfection of M-CAM cDNA into M-CAM-negative breast carcinoma cells results in smaller tumors that have less metastatic potential and are more cohesive (Shih et al., 1997). M-CAM also has an important role in vascular development in zebrafish (Chan et al., 2005) and it has been identified in the somites (Pujades et al., 2002), although its function in myogenic cells remains unclear.

In the current study, we identified proteins whose regulation is modulated during fusion of human fetal mononuclear muscle cells into myotubes. Primary human fetal muscle cell cultures derived from seven individuals were established. Microarray analyses were performed on RNA derived from proliferating myogenic cells and compared with cells undergoing differentiation at two different stages of myotube formation (early and late myotubes). M-CAM was detected among the genes that are highly expressed in human fetal myoblasts and are downregulated during the process of cell fusion. Characterization of M-CAM expression in human fetal muscle demonstrated that this protein is expressed by both endothelial and myogenic cells in vitro and in vivo, as indicated by co-expression of M-CAM with CD31 (endothelial marker) and with Pax7, desmin, M-Cadherin and MyoD (myogenic progenitor markers). Primary human fetal muscle cells fractionated based on the expression of M-CAM alone (M-CAM$^+$) or in conjunction with the endothelial marker CD31 (M-CAM$^+$ CD31$^+$) appear to be myogenic, whereas M-CAM$^-$CD31$^+$ cells are non-myogenic. In vitro RNA-knockdown studies confirmed that myoblasts with decreased M-CAM expression exhibited increased myoblast fusion. These studies demonstrate that M-CAM is a previously unrecognized protein highly expressed on human fetal myogenic cells, and its downregulation enhances fusion of myoblasts into myotubes.

Results

Isolation and characterization of human fetal skeletal muscle

Mononuclear cells dissociated from muscle of individuals aged 13–18 gestational weeks were plated for 2 days in proliferation medium with high serum. At day 2, adherent cells were trypsinized and re-plated at a density of 3–5 × 10^5 cells in 100-mm dishes. To determine the

percentage of myoblasts within each culture, cells were stained for desmin, a marker expressed specifically on muscle precursor cells (Kaufman et al., 1991) around day 3 after explantation. In each sample, the percentage of myogenic cells ranged between 70 and 90% (Fig. 1A–D, Table 1).

To identify genes which are regulated during myoblast fusion, three time points were chosen to harvest cellular RNA for microarray analyses: myoblasts (mononuclear muscle cells maintained in proliferation medium at 50–60% confluence) (Fig. 1A,B), early myotubes that had been placed in differentiation medium for 24–48 hours and contained myotubes with approximately 2–6 nuclei (Fig. 1E, Table 1) and late myotubes that had been placed in differentiation medium for 48–96 hours and contained more than 15 nuclei per myotube (Fig. 1F, Table 1). The ‘early’ and ‘late’ myotube samples naturally contained a variable proportion of mononuclear cells, indicating the ongoing process of cell fusion.

For each of the 21 samples, the extracted total RNA was reverse transcribed and hybridized to Affymetrix U133A gene chips, which interrogate the expression of a total of 14,500 genes (or 18,400 transcripts). Differentially expressed genes in the condition ‘myoblasts’ versus early myotubes and ‘myoblasts’ versus late myotubes were identified using the geometric fold change and Bayesian analysis of differential gene expression (BADGE) analyses. The analyses yielded 230 differentially expressed probe sets when comparing the conditions myoblasts versus early myotubes and myoblasts versus late myotubes (supplementary material Table S1). Several genes known to be upregulated during muscle cell fusion were detected among the differentially expressed transcripts, including troponin, myosin, tropomyosin, MEF2C, ADAM12 and muscle creatine kinase. Among the downregulated genes, transcripts for the hepatocyte growth factor (met proto-oncogene), several inhibitors of DNA binding and vascular endothelial growth factors were selected as differentially expressed in the current analysis (supplementary material Table S1). The differentially expressed genes were further classified by gene ontology using DAVID (http://apps1.niaid.nih.gov/david) (supplementary material Table S2) and include, for example, a number of sarcomeric protein genes (including actin, myosin, troponin), muscle-specific ion channels (i.e. ryanodine receptor R1) and other muscle-specific transcripts among the upregulated genes (i.e. caveolin 3, MEF2C, muscle creatine kinase).

Among the genes that were significantly downregulated in both comparisons of myoblasts versus early myotubes and myoblasts versus late myotubes, the gene melanoma cell adhesion molecule (M-CAM) appeared multiple times (supplementary material Table S1). Three independent probe sets indicated the downregulation of M-CAM mRNA expression during myoblast fusion (supplementary material Table S1). M-CAM was chosen for further analysis for its localization in the membrane, role in cell-cell interaction and migration, and because its expression has been reported in cells located within the somite during development (Pujades et al., 2002). To confirm that expression of M-CAM is downregulated during myoblast cell fusion, quantitative real-time PCR was performed on total RNA derived from all seven human samples (Fig. 2A,B). The internal control GAPDH was co-amplified in each sample and the *C*t values for this reference gene remained constant (Fig. 2A,B), whereas the *C*t value for M-CAM appeared to increase in the early and late myotube samples compared with myoblasts (Fig. 2A), confirming that there is a decrease of M-CAM RNA during the fusion of myoblasts into myotubes (Fig. 2B).

Two splice variants have been described for M-CAM in avian species, a full-length isoform and an isoform lacking exon 15 (Taira et al., 2004). To determine whether multiple isoforms of M-CAM are expressed in human fetal myoblasts, the cytoplasmic region of M-CAM, including exon 15, was amplified by real-time PCR (Fig. 2C). A single 559 bp band corresponding to the full-length isoform was amplified from myoblasts as well as early and

late myotube samples (Fig. 2C) and the identity of this band was confirmed by sequence analysis. Amplification of M-CAM by real-time PCR was also performed on primary myoblasts derived from the quadriceps skeletal muscle of an adult individual, in which 76% of the mononuclear cells were positive for desmin (data not shown). Sequence analysis of the amplified product confirmed expression of the full-length cytoplasmic form of M-CAM in adult myoblasts.

Comparison of MCAM expression levels in adult versus fetal myoblasts

Quantitative real-time PCR and immunohistochemistry analyses were performed in parallel on adult and fetal myoblasts to compare M-CAM expression levels (Fig. 3). Real-time PCR analysis revealed that M-CAM RNA was more abundant in fetal (Fig. 3A) than in adult myoblasts (Fig. 3B), as indicated by the difference in the fold change values (Fig. 3C). This was also confirmed by immunohistochemistry (Fig. 3D–F) performed on cytospins of primary myoblasts derived from fetal and adult individuals. Fetal cells displayed a higher expression of M-CAM protein (Fig. 3E) compared with adult muscle cells (Fig. 3F). These results suggest that M-CAM may play a role in human fetal myogenic cellular differentiation that is not conserved in adult myoblasts.

To confirm that M-CAM-positive mononuclear cells are myogenic and determine their location in vivo, human fetal and adult skeletal muscle tissue sections were analyzed by immunohistochemistry for co-localization of M-CAM expression with other myogenic markers, such as M-cadherin, Pax7 and MyoD (Fig. 4). Tissue sections of human fetal skeletal muscle stained simultaneously for M-CAM and M-Cadherin (Fig. 4A) or M-CAM and Pax7 (Seale et al., 2000;Olguin and Olwin, 2004;Zammit et al., 2004) (Fig. 4B,C) or M-CAM and MyoD (Fig. 4D–F) demonstrated that these markers often, but not always, co-localize to the same cells, indicating that a portion of M-CAM-positive cells in human fetal muscle are likely to be myogenic. In tissue sections, adult mouse skeletal muscle M-CAM-positive cells were detected, but they never expressed the myogenic marker Pax7 (Fig. 4G–H) or MyoD (data not shown).

M-CAM has been reported as a marker expressed by endothelial cells (Anfosso et al., 2001), and it was detected in the current analysis on the surface of cells both negative and positive for myogenic-specific markers. To further investigate whether the M-CAM$^+$, apparently non-myogenic cells were endothelial in origin, co-staining of M-CAM and CD31, an endothelial specific marker, was performed on tissue sections by immunofluorescence and on primary cultures of human fetal muscle cells by fluorescence-activated cell sorter (FACS) analysis. On tissue sections, co-staining of CD31 and M-CAM revealed the presence of M-CAM$^+$ CD31$^-$ cells adjacent to myofibers (Fig. 5A,B open arrowhead) as well as cells that were M-CAM$^+$ CD31$^+$ (Fig. 5B,C solid arrowheads) some of which were clearly located within blood vessels (Fig. 5C).

To determine whether a fraction of CD31-positive cells express myogenic markers, co-staining of CD31 with the myogenic factors MyoD (Fig. 5D–G) and Pax7 (Fig. 5H) were again performed on tissue sections from human fetal skeletal muscle. Results demonstrated the presence of CD31$^+$MyoD$^+$ cells (Fig. 5D–F) and CD31$^+$MyoD$^-$ cells (Fig. 5G). Co-localization of CD31 and Pax7 clearly indicated that these markers are on distinct cells in skeletal muscle (Fig. 5H).

To further address the myogenic potential of cells within human fetal muscle based on M-CAM and CD31 expression, primary cultured mononuclear cells were stained, analyzed, and sorted using the FACS into four distinct groups: MCAM$^+$CD31$^-$, MCAM$^+$CD31$^+$, MCAM$^-$CD31$^+$ and MCAM$^-$CD31$^-$ (Fig. 6A,B). The purity of the sorted cell populations after FACS was estimated to be 85%, leaving the possibility of few cross-contaminant cells in any given

fraction. All sorted cell populations were cultured in equal numbers (15,000 cells) overnight in growth medium. After overnight plating, $MCAM^{+}$ $CD31^{-}$ (Fig. 6C) $MCAM^{+}CD31^{+}$ (Fig. 6F) and $MCAM^{-}CD31^{-}$ (Fig. 6L) cells appeared to have adhered to the plate. By contrast, the majority of $MCAM^{-}CD31^{+}$ cells appeared round and non-adherent (Fig. 6I). Desmin immunostaining revealed that $MCAM^{+}CD31^{-}$ (Fig. 6D) and $MCAM^{+}CD31^{+}$ (Fig. 6G) contained a high proportion of desmin-positive cells whereas the majority of $MCAM^{-}CD31^{+}$ (Fig. 6J) and $MCAM^{-}CD31^{-}$ (Fig. 6M) cells did not express desmin. Quantification of the percentage of desmin-positive cells in ten random fields of view for each of the four sorted cell populations revealed a significantly higher number of myogenic cells in the fractions expressing M-CAM alone or together with CD31 than those lacking M-CAM expression (Fig. 6O). The four cell fractions were also placed in differentiation medium and the ability to form myotubes was quantified by assessment of the fusion index (Fig. 6P). As predicted from the percentage of desmin-positive cells contained in each fraction, $MCAM^{+}CD31^{-}$ and $MCAM^{+}CD31^{+}$ contained the highest number of myotubes (Fig. 6P) that expressed myosin heavy chain (MHC) (Fig. 6E,H). These results demonstrate that fractionation of human fetal skeletal muscle primary cells based on the expression of the cell surface marker M-CAM significantly enriches for myogenic cells.

Role of M-CAM in myoblast fusion assessed by RNAi

To investigate the function of M-CAM and to determine whether downregulation of its expression enhances fusion of myoblasts into multinucleated myotubes, the M-CAM message was knocked down in vitro using RNA interference technology (RNAi). C2C12 is a well-studied murine muscle cell line that reliably mimics the differentiation steps occurring in primary muscle cells in vitro (Yaffe and Saxel, 1977). Analysis of M-CAM expression on RNA extracted from proliferating C2C12 cells was performed using quantitative real-time PCR. Amplification of the cytoplasmic region of murine M-CAM revealed the presence of two bands (data not shown). Upon sequence analysis, these bands corresponded to two splice variants of M-CAM, one full-length transcript and one smaller transcript lacking exon 15, contained in the cytoplasmic tail of M-CAM. RNAi knockdown experiments on C2C12 cells were designed to deplete the expression of both M-CAM isoforms. Small hairpin RNAs (shRNAs) were generated using oligonucleotide 20, which is specific to exons 5–6 and oligonucleotide 38, targeting exons 8–9 of murine M-CAM. All these exons are located within the extracellular domain of M-CAM (Taira et al., 2004). C2C12 myoblasts were transduced with pSIREN-retroQ20 (Fig. 7D), pSIREN-retroQ38 (Fig. 7F) or with p-SIREN-retroQNeg as negative control (Fig. 7B). To confirm that M-CAM RNA and protein expression were downregulated in C2C12 cells transduced with oligo 20 and 38 compared with control cultures, quantitative real-time PCR (Fig. 7G) and immunohistochemistry analyses (Fig. 7A,C,E) were performed on the three cell lines. Quantitative real-time PCR of M-CAM RNA in the cell line transduced with oligo 20 resulted in expression of M-CAM RNA at 40% of normal levels, whereas cells transduced with oligo 38 resulted in expression of only 13% of M-CAM RNA levels compared with the negative control (Fig. 7G). Cell samples from all cultures were also tested for remaining M-CAM protein expression by immunohistochemistry (Fig. 7A,C,E). Photographs were acquired at the same exposure in all cultures to allow for visual comparisons. The negative-control cells retained M-CAM expression (Fig. 7A), whereas the two cell lines infected with oligo 20 (Fig. 7C) or oligo 38 (Fig. 7E) expressed lower or undetectable amounts of M-CAM. Both RNA and protein results demonstrated that the cells transduced with oligo 38 resulted in a more robust downregulation of M-CAM expression. Western blot analyses were also attempted to determine the amount of M-CAM protein in each cell line. However, the anti-M-CAM antibody failed to detect the target protein under denaturing conditions (data not shown).

As gene expression studies had indicated that expression of M-CAM should be downregulated for muscle cells to fuse, C2C12 cells transduced with either 'negative' vector, oligo 20 or oligo 38 were tested in parallel for any ability to differentiate into multinucleated myotubes. Ten thousand C2C12 cells derived from each RNAi treatment were placed in differentiation medium and after 3, 7 and 12 days the fusion index was calculated for the three different cell populations by counting the nuclei of cells fused in myotubes over the total number of nuclei in multiple random microscopic fields (Fig. 7B,D,F). At day 3, the averaged fusion index was 13% for the negative control, 17% for the culture treated with oligo 20 (which resulted in 40% expression of M-CAM RNA) and 24% fusion index for the culture treated with oligo 38 (that resulted in 13% expression of M-CAM RNA compared with normal amounts) (Fig. 7H). The fusion index increased in all cultures at day 7, but it remained higher for the cultures treated with oligos 38 and 20 compared with the control. At day 12, all cultures had a similar fusion index (Fig. 7H). Statistical comparison of the fusion indexes using a Wilcoxon rank sum test of the control culture versus the M-CAM oligo 20-treated culture yielded a P value of 0.03 at day 3 and a P value of 0.01 at day 7, whereas comparison of control and the M-CAM oligo 38-treated culture gave a $P<0.002$ at both days 3 and 7. At day 12 no significant P values were obtained. These results demonstrate that decreased expression of M-CAM in muscle cells in vitro enhances their fusion into multinucleated myotubes.

Discussion

During development, mature muscle is formed through fusion of mononuclear cells into multinucleated myofibers. Myofibers undergo mechanical stress during contraction and relaxation which may result in local sarcolemmal injury. When myofibers are in need of repair, fusion of mononuclear cells (i.e. satellite cells) is believed to occur at the site of membrane tear. Thus, cellular fusion in muscle is a key mechanism that contributes not only to muscle formation but also to maintenance during postnatal life. Furthermore, for optimization of cell-based therapy of muscle diseases, mononuclear cells are required to fuse and become part of pre-existing myofibers. Hence, for muscle development and homeostasis, and for therapeutic applications, cell fusion is a key process. Muscle cell differentiation can be recapitulated in vitro by expanding dissociated muscle precursors in the presence of high serum and mitogens, whereas low-serum concentrations induce fusion of proliferating cells into multinucleated myotubes (Bischoff and Holtzer, 1969; Richler and Yaffe, 1970; Hartley and Yablonka-Reuveni, 1990; Rando and Blau, 1994; Yablonka-Reuveni and Rivera, 1994). Accordingly, in vitro differentiation of muscle cells is a good model for studying proteins that are regulated during cellular fusion.

In the current study, human fetal muscle cells cultured under proliferative conditions in vitro were induced to differentiate into multinucleated myotubes, harvested and analyzed using microarrays to identify genes that are significantly up- or down-regulated during fusion.

Among the genes identified in the current analysis, we focused on melanoma cell adhesion molecule (M-CAM), a transmembrane protein that appears to be highly expressed on proliferating myogenic cells and is significantly downregulated during muscle cell fusion. M-CAM is known to be involved in metastasis formation in cancer (Mills et al., 2002), but little is known about its function in muscle. During chick development, M-CAM RNA is expressed in the dorsal part of the somite, known as the myotome (Pujades et al., 2002). In addition, expression of M-CAM RNA is also increased during conversion of the mesoderm-like 10T1/2 cell line to muscle cells after exposure to 5-azacytidine (Pujades et al., 2002). Although these observations collectively indicate that M-CAM might be a protein expressed during myogenesis, little is known about its conservation, expression pattern and putative function in muscle cells from other species. Our studies demonstrate that M-CAM is expressed on human fetal myogenic cells and its downregulation appears necessary to promote cell fusion. On

cytospins of cultured cells, M-CAM was co-expressed with the myoblast-specific markers desmin and M-cadherin, whereas on tissue sections M-CAM co-localized with cells positive for the myogenic markers Pax7 (Seale et al., 2000; Olguin and Olwin, 2004; Zammit et al., 2004) and M-cadherin (Irintchev et al., 1994; Sajko et al., 2004). Collectively, these results suggested that M-CAM may be a novel marker for activated satellite cells, because it appears to be expressed by cells weakly positive for Pax7 (Zammit et al., 2004) and on cultured satellite cells expressing desmin and M-cadherin (Cornelison and Wold, 1997).

M-CAM was detected on cells with myogenic potential that also express endothelial markers. By FACS analysis, human fetal muscle mononuclear cells were fractionated based on expression of M-CAM and the endothelial marker CD31. Subsequent analyses by immunofluorescence demonstrated that all cells positive for M-CAM, including cells positive for both M-CAM and CD31 are highly myogenic, as indicated by desmin expression, high fusion index compared with M-CAM$^-$cells, and myosin heavy chain expression in formed myotubes. Although the majority of satellite cells in limb muscles appear to be somitic in origin (Gros et al., 2005; Relaix et al., 2005), studies have also highlighted the existence of myogenic cells derived from the dorsal aorta that express endothelial markers (De Angelis et al., 1999). Thus, it is possible that M-CAM$^+$CD31$^+$ cells with myogenic potential detected in human fetal muscle in the current study may be related to these myoendothelial precursors (Cossu and Biressi, 2005). Interestingly, M-CAM expression on myogenic cells appears to be high in human primary fetal cells and significantly decreased in adult cells. Although the precise reason for this downregulation is not understood, one possibility is that M-CAM in fetal cells may mediate migration of myogenic precursors from other sites, like the somites or dorsal aorta, to the limb musculature.

The current study also demonstrates that downregulation of M-CAM in vitro induces myoblast fusion and formation of multinucleated myotubes. Microarray analyses interrogating genes that are significantly up- or downregulated during myoblast fusion indicated that M-CAM is a strongly downregulated gene. These results were confirmed both by quantitative real-time PCR and immunohistochemistry analyses. Studies on lymphocytes have demonstrated that regulation of cell adhesion molecule (CAM) and integrin expression is crucial for trafficking and homing of cells in various tissues (Papayannopoulou, 2000; Bonig et al., 2006). Interestingly, our microarray analyses detected a significant increase in α_4 integrin (VLA-4) during early and late myotube fusion, concomitant with M-CAM downregulation. Although not proven, it is possible that M-CAM downregulation and VLA-4 upregulation may play a synergistic role during muscle cell fusion. An involvement of VLA-4 interaction and signaling during myoblast fusion has been previously reported (Rosen et al., 1992), however subsequent studies on VLA4-knockout mice did not show any apparent phenotypic variation in muscle cell fusion in vitro or in the musculature in vivo (Yang et al., 1996). Therefore, the exact role exerted by VLA4 alone, or together with other molecules, on muscle cells and how this may affect cellular fusion is still unclear. Our study demonstrates that expression of M-CAM in human fetal muscle is associated with myogenic and myoendothelial cells. In addition, we demonstrated that downregulation of M-CAM increases myoblast fusion. Although these results suggest that M-CAM may be involved in muscle cell proliferation, stimulation of cultured cells with HGF failed to increase the percentage of cells positive for M-CAM (data not shown). Other mitogens and growth factors not tested in the current study may stimulate and sustain M-CAM expression during myoblast proliferation, however, it is also possible that the role of M-CAM in human fetal muscle cells is unrelated to cell proliferation. Future studies will be necessary to elucidate these intriguing putative roles of M-CAM in muscle cells.

Materials and Methods

Tissue preparation

Mononuclear cells were isolated from de-identified, discarded human fetal muscle samples of gestational age 13 to 18 weeks (Advanced Bioscience Resources, Alameda, CA and from the University of Washington). These samples were collected under protocol S05-01-010, approved by the Committee of Clinical Investigation at Children's Hospital Boston. Samples were collected in sterile HBSS 1% glucose, 1% penicillin-streptomycin and shipped on wet ice. Skeletal muscle was minced into fine pieces and enzymatic dissociation was performed in collagenase (0.5 mg/ml) (Worthington, Lakewood, NJ) dispase II (0.6 U/ml, Roche Applied Science, Indianapolis, IN) for 45–75 minutes at 37°C as described (Pavlath and Gussoni, 2005). Samples were then filtered through a 40-μm cell strainer before cell suspensions were cultured on plastic plates (Costar, Corning Incorporated, NY) in proliferating medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO Invitrogen Corporation, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), epidermal growth factor (EGF; 1 ng/ml; Atlanta Biologicals, Lawrenceville, GA) and basic fibroblast growth factor (bFGF; 1 ng/ml; Atlanta Biologicals, Lawrenceville, GA). Cells were maintained for 2 days in proliferation medium with high serum, after which they were trypsinized and re-plated at a density of $3–5 \times 10^5$ cells in 100-mm dishes. Cells were grown in proliferation medium and passaged when approximately 60% confluent. To assess the percentage of myogenic cells within each sample, desmin staining was performed on day 3 after explantation in cells maintained in proliferation medium. To induce myogenic differentiation, cells were exposed to low-glucose DMEM supplemented with 4% FBS serum for 2–4 days.

RNA isolation and cDNA microarray

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was extracted from cells derived from seven individuals and cultured under three separate conditions: proliferation medium (myoblasts), early myotubes (myotubes containing to 2–6 myonuclei) and late myotubes (myotubes containing more than 15 myonuclei) (n=21 samples). Each individual target cDNA was synthesized from 7 μg total RNA using SuperScript double-stranded cDNA synthesis (Invitrogen, Carlsbad, CA) and cRNA was labeled using Enzo RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). 20 μg of fragmented, biotin-labeled cRNA was prepared and hybridized onto the Affymetrix human HG-U133A GeneChip. Each microarray was optically scanned and the resulting image was processed using the Affymetrix GeneChip MAS5.0 analysis software to quantify the hybridized RNA transcript levels that were then summarized in a CHP file. Each surveyed gene transcript was given an average fluorescence intensity value (Signal) indicating the amount of the transcript detected and a call (Present, Absent or Marginal) indicating the robustness of its detection.

Microarray data analysis

Each Affymetrix HG-U133A microarray contains 22,283 individual probe sets representing 18,400 RNA transcripts and variants, including 14,500 well-characterized human genes. The recorded expression values ranged between 0.20 and 128899.60. Only those genes with at least one measurement above 20 were considered. As a result, a total of 22,266 probe sets were included in the analysis. Data were analyzed using a geometric fold-change analysis (Haslett et al., 2002; Lennon et al., 2003; Sanoudou et al., 2003; Liadaki et al., 2005) and using BADGE version 1.0 (Bayesian Analysis of Differential Gene Expression http://genomethods.org/badge/). BADGE is a computer program implementing a Bayesian approach to identify differentially expressed genes across experimental conditions. For each gene, the method computes the posterior probability that the gene is expressed more than one

fold in the first condition than in the second condition. A predictive evaluation of the results obtained by this method was performed using 'leave-one-out' cross validation.

PCR analyses

Real-time quantitative PCR of M-CAM on human fetal muscle cDNA—To confirm the results obtained by gene expression analyses, independent validation of mRNA expression was performed via real-time quantitative PCR on genes of interest. 2 μg of total RNA extracted from the original cell preparations used for microarray analyses was reverse-transcribed using the TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a 100 μl reaction volume according to manufacturer's protocol. Samples were assayed using the SYBR GREEN PCR Master Mix (Applied Biosystems Foster City, CA). Sense and antisense primers were used at a final concentration of 300 nM with the primers for human M-CAM sense, 5′-CTGCTGAGTGAACCACAGGA-3′ and antisense, 5′-TCAGGTCATGCAACTGAAGC-3′. The primers used for the GAPDH control were sense, 5′-GAAGGTGAAGGTCGGAGTC-3′ and antisense, 5′-GAAGATGGTGATGGGATTTC-3′. Samples were amplified for 46 cycles under the following conditions: denaturation 95°C for 15 minutes, annealing and extension 60°C for 1 minute. Data analysis was performed using the Sequence Detector v1.7a software. All quantifications were normalized to the endogenous control GAPDH to account for variability in the initial concentration, quality of the total RNA and for the conversion efficiency of the reverse transcription reaction.

M-CAM mRNA amplification and sequencing—The cytoplasmic regions of murine and human M-CAM were amplified by PCR using species-specific primers. The oligonucleotides used for mouse M-CAM were sense AGTGTACAGCCTCCAAC (exon 12), antisense ATGCCTCAGATCGATG (exon 16). For human M-CAM, the sense primer CCATCTCCTGGAACGTCAAC (exon 12) and antisense TAATGCCTCAGATCGATGTATTTC (exon 16) were chosen. Samples were amplified for 46 cycles under the following conditions: denaturation 95°C for 15 minutes, annealing and extension 60°C for 1 minute. Amplified PCR products were purified before being sequenced using the Exosapit kit (USB Corporation, Cleveland, OH). Sequence analysis was carried out using the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Quantification of M-CAM RNA on RNAi cell lines—To determine the amount of M-CAM RNA in the siRNA C2C12 cell lines, cDNA synthesis and multiplex real-time quantitative PCR were carried out using the TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA) on an ABI Prism 7900 Sequence Detector System as previously described (Tomczak et al., 2004). cDNA for each siRNA primer replicate was synthesized in duplicate for a total of six cDNA samples per siRNA primer. In every 20 μl reaction, 10 μl Universal Master Mix (Applied Biosystems, Foster City, CA), 1 μl cDNA, 1 μl M-CAM1 (Mm00522397_m1) Assays-on-Demand primer set and 1 μl 18S rRNA primer set (Applied Biosystems) were included. Data analysis was performed using the Comparative delta Ct Method as described in the manufacturer's manual (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, Applied Biosystems).

Immunohistochemistry

For cells in culture, four-well chamber slides were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Slides were washed in PBS 0.5% Triton X-100 for 3 minutes, then blocked for 30 minutes at room temperature in 10% FBS diluted in PBS 0.1% Triton X-100. Mouse anti-CD146 (Chemicon, Temecula, CA; 1:500), mouse anti-Pax7 (DSHB, Iowa City, IA; 1:100), mouse anti-myosin heavy chain monoclonal antibody (DSHB; 1:20), mouse anti M-cadherin (BD Pharmingen, San Jose, CA; 1:50), goat anti-M-

CAM (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), mouse anti-desmin or mouse anti-CD31 (Dako, Carpinteria, CA; 1:50) were diluted in blocking solution, added to the slides and incubated overnight at 4°C in a humid chamber. Slides were washed three times for 10 minutes in PBS on a shaker, incubated with secondary antibodies (anti-mouse FITC or Rhodamine, or anti-goat FITC or Rhodamine; Jackson ImmunoResearch, West Grove, PA; 1:100) for 1 hour at room temperature and washed again in PBS before adding coverslip with Vectashield containing 200 ng/ml DAPI (Vector Labs, Burlingame, CA).

For immunohistochemistry on cytospins of murine C2C12 cells and for staining of human fetal skeletal muscle tissue sections, slides were washed three times with PBS, fixed and blocked as described for cells in culture and incubated with mouse anti-human desmin monoclonal antibody (Dako, Carpinteria, CA), mouse anti-M-CAM monoclonal antibody (Chemicon, Temecula, CA), mouse anti M-cadherin monoclonal antibody 1:100 (BD Pharmingen, San Diego, CA), mouse anti-Pax7 1:100 (DSHB, Iowa City, IA) mouse anti-MyoD 1:100 (C-20 Santa Cruz), mouse anti-CD31 1:100 (Dako, Carpinteria, CA; 1:50), mouse anti-N-CAM 1:100 (AbCam, Cambridge, MA) and incubated overnight at 4°C. Slides were washed, incubated with donkey anti-mouse FITC or Rhodamine (Jackson ImmunoResearch, West Grove, PA) and processed as described for cells in culture.

Flow cytometry analyses

Human fetal muscle cells grown on gelatin-coated plastic plates for 24 hours were washed three times in PBS and lifted using trypsin-EDTA (Sigma Aldrich, St Louis MO). Cells were spun at 365 ***g*** for 10 minutes and resuspended in 1 ml sterile PBS 0.5% BSA. 200 μl of the cell suspension was placed on ice and used as a negative control. Mouse anti-CD146 (Chemicon, Temecula, CA; 1:50) was added and cells were incubated for 25 minutes on ice. Cells were washed and resuspended in 500 μl PBS containing 0.5% BSA and incubated with a secondary anti-mouse FITC antibody (Jackson ImmunoResearch, West Grove, PA; 1:100) for 15 minutes on ice. The negative control sample was also incubated in parallel with the secondary antibody. For co-detection of M-CAM and CD31 by FACS, samples were washed in cold PBS, resuspended in 500 μl PBS-0.5% BSA and incubated for 25 minutes on ice with mouse anti-human CD31-PE (sample previously incubated with M-CAM antibody) or with an isotype control IgG-PE (negative-control sample). Cells were then washed and filtered through a 40 μm filter before being resuspended in 1 ml cold PBS-0.5% BSA containing 2 μg propidium iodide for dead-cell exclusion. Flow cytometry analyses and cell sorting were performed on a dual laser FACSvantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For each sample analyzed, 20,000 total cell counts were acquired using CellQuest software, version 3.3 (Becton Dickinson) and then analyzed using FlowJo version 6.1.1 (Treestar Inc, Ashland, OR).

Labeled cells were sorted into four groups: $MCAM^{+}CD31^{-}$, $MCAM^{+}CD31^{+}$, $MCAM^{-}CD31^{+}$, $MCAM^{-}CD31^{-}$ and 15,000 cells from each group were plated into four-well chamber slides coated with 0.15% gelatin. Cells were grown in the proliferation medium described above for 24 hours and then stained by immunohistochemistry for desmin as described above.

RNA interference experiments

Design of small interfering RNA targeting vectors—Mouse M-CAM cDNA sequence (NM_023061) was analyzed using BD Biosciences Clontech RNAi Designer (http://bioinfo.clontech.com/rnaidesigner/) and Invitrogen's BLOCK-iT RNAi Designer (https://rnaidesigner.invitrogen.com/rnaiexpress/). Two target sequences that were found on both lists and ranked by Invitrogen software with the highest knockdown probability, were selected (M-CAM oligo 20 GAGGAGGAGAACCGAGTTC targeting murine M-CAM exons 5–6 and M-CAM oligo 38 CTATGTGTCTGATGTTCAA targeting exons 8–9). They were

subjected to an NCBI blast search (www.ncbi.nlm.nih.gov/BLAST/) and did not show homology to any other mouse genes or ESTs. The Clontech siRNA Hairpin Oligonucleotide Selector was used to generate the sequence of two complementary oligonucleotides suitable for cloning into the pSIREN RetroQ siRNA expression system (BD Clontech, Mountain View, CA). This vector contained the Human U6 RNA polymerase III promoter and it also expressed a puromycin-resistance gene enabling selection of infected cells. Each siRNA consisted of a 19-base-pair sequence corresponding to the sense strand of the targeted RNA and a 19-base-pair anti-sense strand, separated by a short 6-base-pair sequence. A negative control vector, which expressed a short RNA sequence that did not form a hairpin, served to control for retroviral infection and transcription from the U6 promoter. Oligonucleotides 20 and 38 were annealed according to instructions in the BD Knockout RNAi Systems User Manual (BD Biosciences, PT3739-1) and ligated into pSIREN RetroQ vector using T4 DNA Ligase (Invitrogen). Ligation products were transformed into One-Shot TOP10 Chemically Competent E.Coli cells (Invitrogen) and grown on agar plates containing 100 μg/ml ampicillin (Sigma Aldrich, St Louis, MO) and incubated overnight at 37°C. Single bacterial colonies were grown in 5 ml LB media supplemented with 100 μg/ml ampicillin and plasmid DNA was extracted using the QiaPrep Spin Miniprep Kit (Qiagen, Valencia, CA). Restriction digest analysis with *MluI* was used to identify bacterial cultures with a properly cloned plasmid. These bacterial cultures were inoculated in 500 ml of LB and grown overnight at 37°C; plasmid was extracted from these cultures using a Qiagen Maxiprep Kit.

Production of retrovirus—1×10^6 Hek293 EcoPack packaging cells (BD Biosciences) were plated in each well of a six-well (9.4 cm^2) collagen-coated plate. Cells were incubated at 37°C in 1.5 ml of DMEM-10% FCS with 2 mM L-Glutamine, without antibiotics. For each transfection, 4 μg retroviral plasmid was diluted in 300 μl Opti-MEM I (Invitrogen Gibco, Grand Island, NY), mixed with 20 μl Lipofectamine 2000 (Invitrogen) in 300 μl Opti-MEM I, incubated at room temperature for 20 minutes and then added to the cell culture. After 4 hours, penicillin and streptomycin supplemented media were introduced. All transfections were performed in triplicate, and three mock transfections were also included. Media was changed the following day. After 48 hours of incubation, media was collected from the cell cultures and filtered through a 45 μm cellulose acetate filter (VWR). The filtrate was used for retroviral infection of C2C12 cell cultures.

Retroviral infection and selection of stable cell cultures—5×10^4 C2C12 cells were plated per well of a six-well plate in growth media. 900 μl of retroviral filtrates (of M-CAM 20, 38, negative control and mock) were mixed with 100 μl FCS and 8 μg Polybrene (Sigma Aldrich, St Louis, MO) and added in each well. Cells were incubated for 15 minutes at 37°C and then spun at 1015 ***g***. The infection media was replaced with fresh growth media and the cells were washed once in 1× PBS and split into two 100-mm dishes in growth media supplemented with 2.5 μg/ml puromycin (BD Biosciences). Medium was changed every other day for 7 days until all of the cells from the mock-transfected wells died. Newly generated cell lines were plated at a density of $10^4/cm^2$ into 100-mm diameter dishes. 24 hours later cells showed 40–50% confluency. After 72 hours, cells (90–100% confluent) were switched into differentiating medium (DMEM with 2% horse serum) that was subsequently changed every 24 hours. RNA from day 0 was harvested using the RNeasy Kit (Qiagen, Valencia, CA) and processed independently from three replicate plates.

Measurements of fusion indices—Six-well plates (Nalge Nunc International, Rochester, NY) of C2C12 cells at differentiation day 2, 7 and 12 were washed twice with 1× PBS and fixed in ice-cold methanol for 10 minutes. After two brief washes in PBS, cells were incubated in 2 μg/ml DAPI in PBS for 5 minutes at room temperature. Staining for expression of myosin heavy chain was performed using immunofluorescence as described above. Cells were

visualized using the 20× objective on a Nikon Eclipse TE2000-S microscope and photographed using a Spot 7.4 slider camera. The images were processed using Spot software version 4.0.9. (Diagnostic Instruments, Sterling Heights, MI). Photographs were taken randomly. Two pictures were taken for each view: a phase-contrast image to visualize cell morphology and a fluorescent image to visualize DAPI stained nuclei and MHC-positive myotubes. These images were used to count the number of nuclei in multinucleated myotubes and the total number of nuclei in the frame. The percentage of nuclei within myotubes over the total number of nuclei was defined as the 'fusion index'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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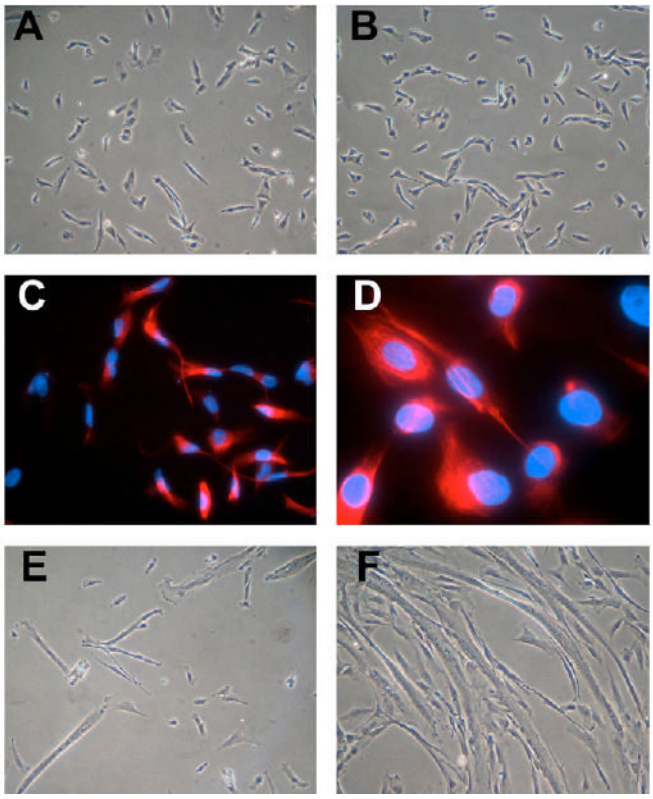

Fig. 1.

Cultures of human fetal cells used for microarray analyses. (A,B) Primary myoblast cultures from sample 7 and 2, respectively. (C,D) Immunofluorescence staining of myoblasts (sample 2) using an anti-desmin antibody (red), with nuclei counterstained with DAPI (blue). (E,F) Examples of ‘early’ (E) and ‘late’ (F) myotubes at the time of RNA harvesting for microarray analyses (sample 2). Magnification, 200× (A,B,E,F); 400× (C); 630× (D).

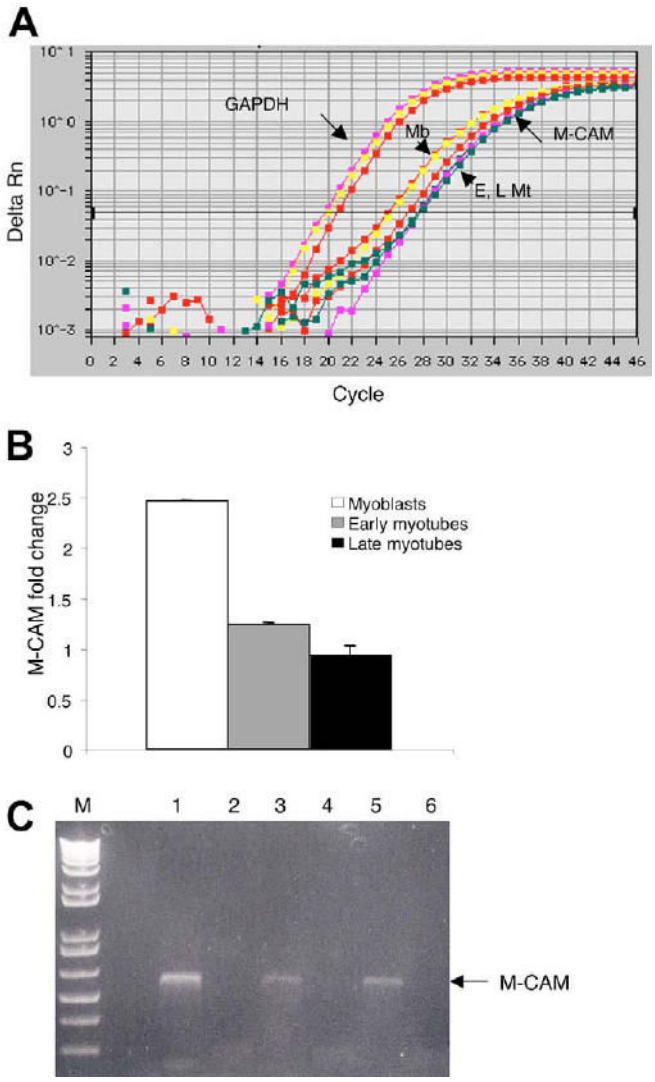

Fig. 2.

(A) Quantitative real-time PCR to assess M-CAM expression in fetal tissue. The different amplification curves demonstrate diverse M-CAM cDNA content at the three time points. Mb, myoblasts; E, L Mt, early and late myotubes. GAPDH was used as internal control. Each PCR reaction was performed in duplicate. (B) Downregulation of M-CAM RNA during myoblast fusion as determined by real-time quantitative PCR analysis. The fold change value (*y* axis) in the late myotubes sample was arbitrarily set as 1 and the fold change values in early myotubes and myoblasts were plotted accordingly. (C) Agarose gel of PCR products after amplification. Sequence analysis confirmed that the band corresponds to M-CAM. M, molecular size markers. Lane 1, myoblasts; lane 3, early myotubes; lane 5, late myotubes; lanes 2, 4, and 6 are water controls for each reaction.

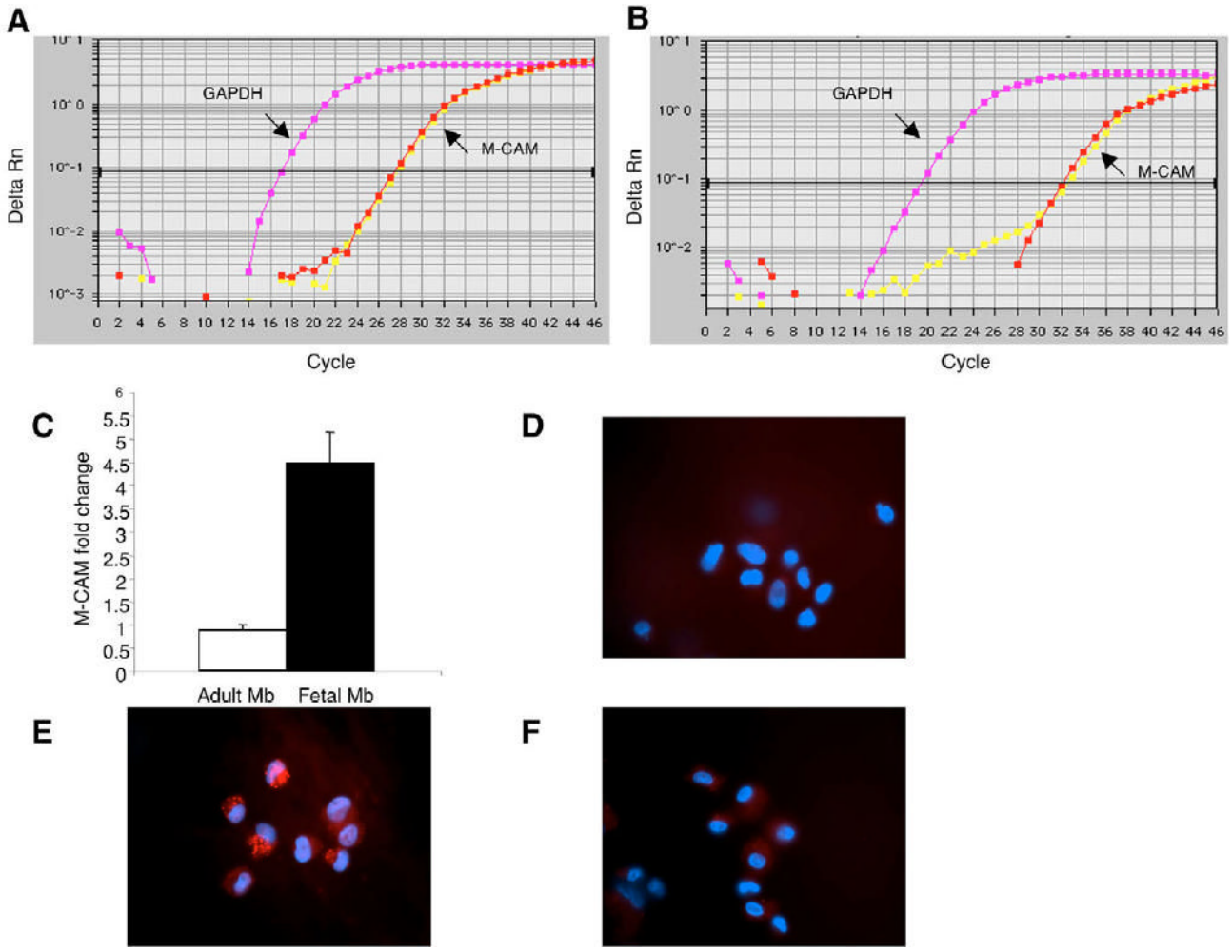

Fig. 3.

Assessment of M-CAM expression in human fetal and adult-derived muscle cells. Quantitative real-time PCR for M-CAM cDNA in human fetal- (A) and adult (B)-derived muscle cells. GAPDH was used as an internal control. (C) Fold change in M-CAM expression in fetal and adult myoblasts. The difference in delta *C*t between fetal versus adult M-CAM cDNA is 2.5, which translates to ~5-fold upregulation of M-CAM transcript in human fetal cells compared with adult cells. Mb, myoblast. (D–F) Immunofluorescence staining for M-CAM (red) on cytospins of cultured fetal (E) or adult (F) muscle cells. Nuclei are counterstained with DAPI (blue). (D) Negative control (secondary antibody alone) of fetal muscle cells. All images were obtained at the same exposure to allow visual comparisons between samples. Magnification, 400× (D–F).

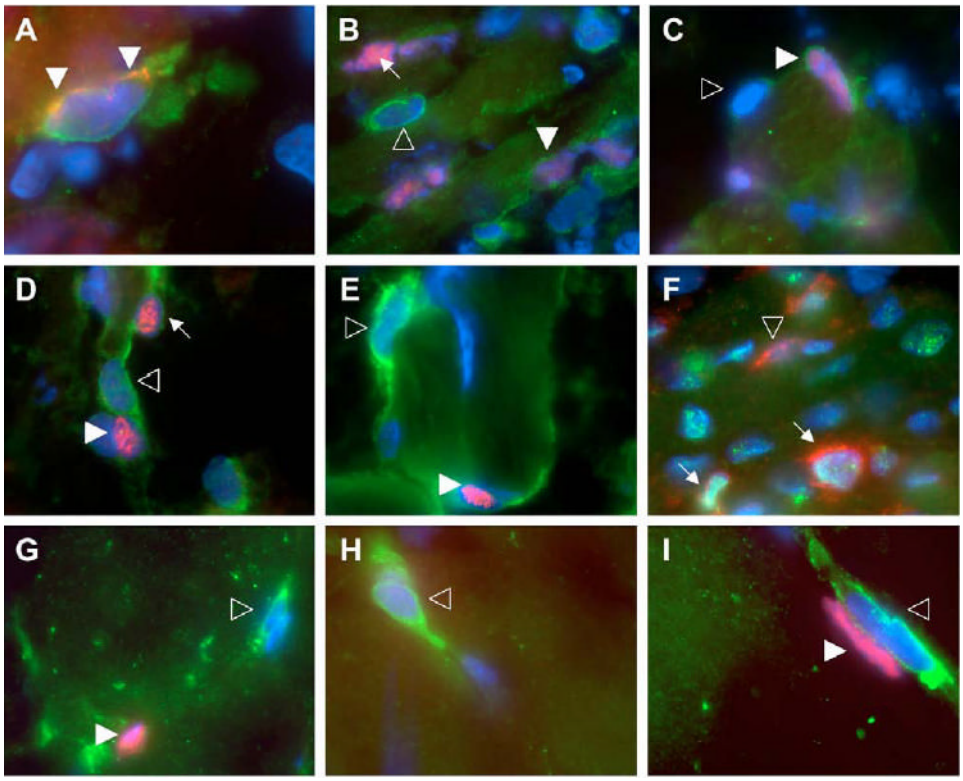

Fig. 4.

Co-detection of M-CAM with myogenic markers on tissue sections from 17-week human fetal muscle and from adult mouse muscle. (A) Co-expression of M-CAM (green) and M-cadherin (red, arrowheads) on a myogenic cell. (B,C) Expression of M-CAM (green) with Pax7 (red). Open arrowheads indicate M-CAM^{+} cells and filled arrowheads M-CAM-Pax7 double-positive cells. Arrow indicates a M-CAM^{-}Pax7^{+} cell. (D–F) Expression of M-CAM with MyoD. (D,E) M-CAM is detected in green and MyoD is in red. (F) M-CAM is in red and MyoD is in green. Note the presence of M-CAM^{+}MyoD^{+} cells (arrows), M-CAM^{+}MyoD^{-} cells (open arrowheads) and M-CAM^{-}MyoD^{+} cells (filled arrowheads). (G–I) Adult mouse skeletal muscle tissue sections stained with M-CAM (green, open arrowheads) and Pax7 (red, filled arrowheads). Note that Pax7 and M-CAM never co-localize to the same cell. Nuclei are stained in blue with DAPI. Magnification, 1000× (A,I); 630× (B–H).

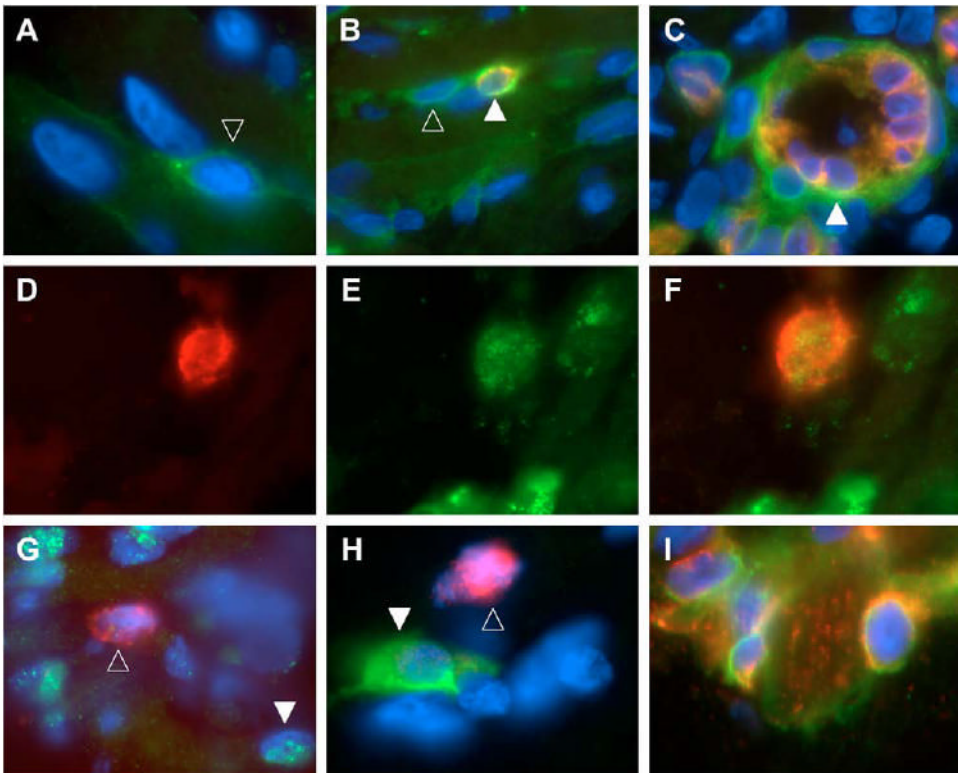

Fig. 5.

Co-expression of endothelial and myogenic markers in tissue sections from human fetal skeletal muscle. (A–C) Expression of M-CAM (green) with the endothelial cell marker CD31 (red). Open arrowheads indicate M-CAM^{+}CD31^{-}cells and filled arrowheads M-CAM^{+}CD31^{+} cells. (D–F) Images from the same field demonstrating the co-expression of CD31 (red) with MyoD (green) (F is the merged image of D and E). (G) CD31 (red, open arrowhead) positive and MyoD (green, closed arrowhead) positive cells. Cells can be CD31^{+}MyoD^{-}. (H) CD31 (green) and Pax7 (red) are expressed by different cells and never co-localize. (I) Co-localization of M-CAM (red, open arrowhead) with N-CAM (green, filled arrowhead). Nuclei are stained in blue with DAPI. Magnification, 1000× (A,D–F,H,I); 630× (B,C,G).

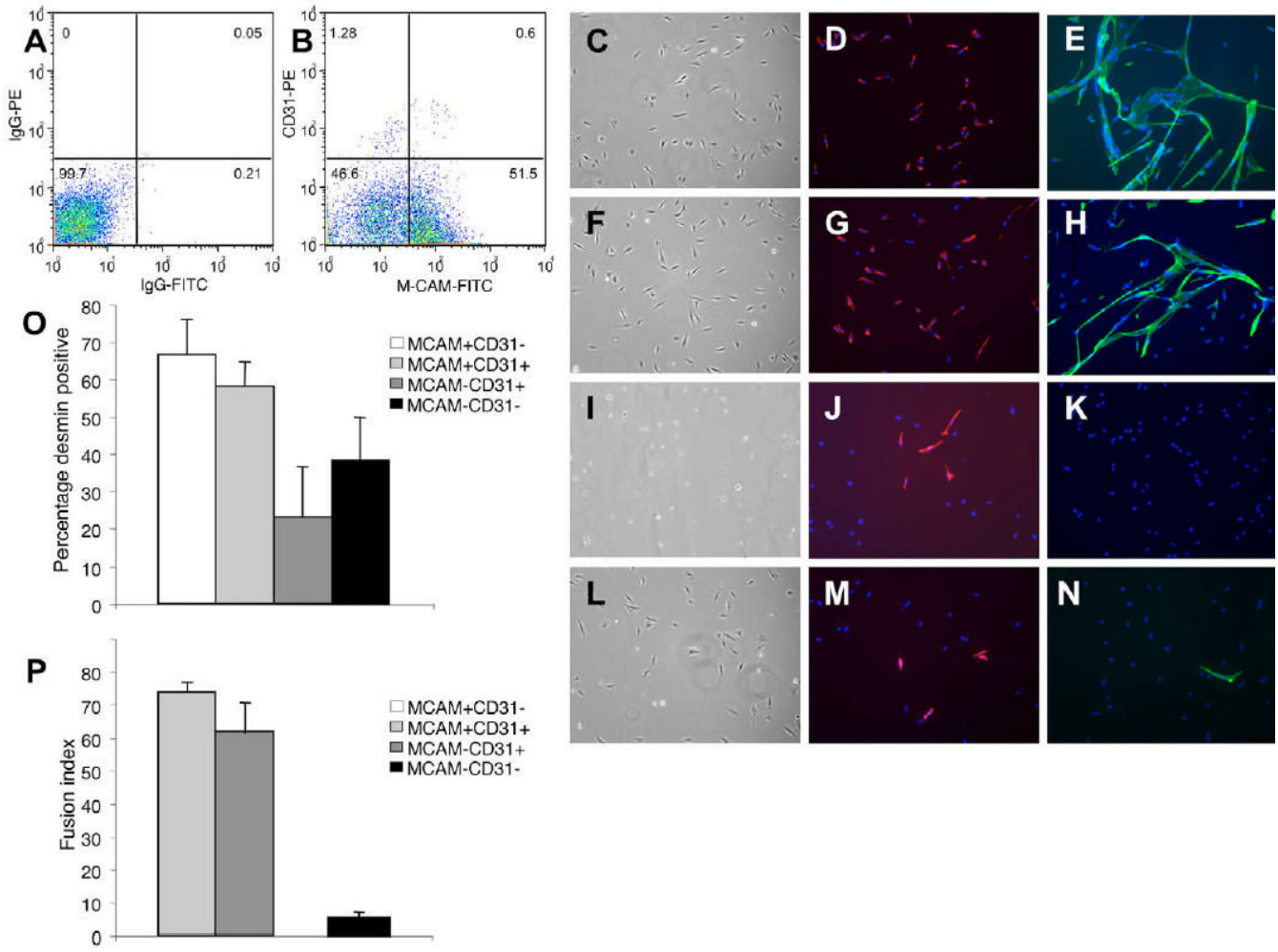

Fig. 6.

Evaluation of myogenic potential of cells fractionated by M-CAM and CD31 expression. (A) FACS analysis of negative control used to set up the gates for cell fractionation. 99.7% of human fetal cells are double negative. (B) M-CAM and CD31 expression in human fetal primary muscle cells. 51.5% of cells are M-CAM-positive, 0.6% are double positive, 1.28% are CD31-positive and 46.6% are double negative. Cells were fractionated according to the displayed gates. (C-N) Brightfield and immunofluorescence images of sorted human cell populations stained for the myogenic marker desmin (red) and myosin heavy chain (MHC, green) after differentiation. Nuclei are stained in blue with DAPI. (C,D,E) M-CAM^{+}CD31^{-} cells; (F,G,H) M-CAM^{+}CD31^{+} cells; (I,J,K) are M-CAM^{-}CD31^{+}; (L,M,N) are M-CAM^{-}CD31^{-}. Magnification, 100×. (O) Percentage of desmin-positive cells in fractionated cell populations as determined by counts performed in ten random fields of view. (P) Fusion index of the fractionated cell populations confirming that cells expressing M-CAM are highly myogenic.

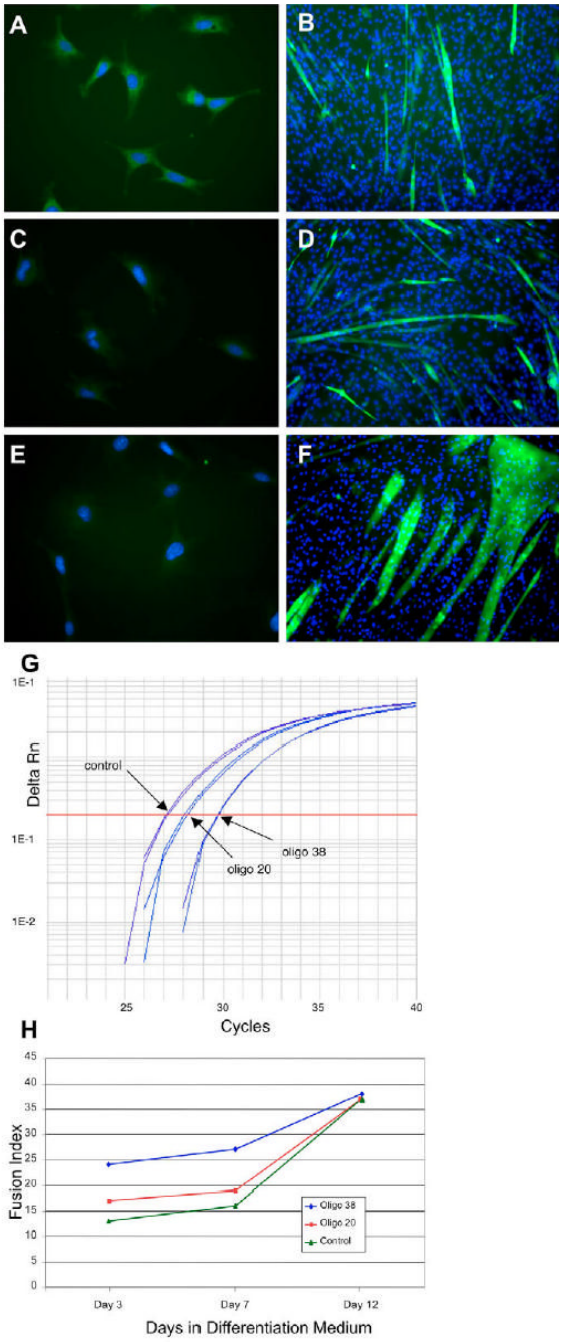

Fig. 7.

Downregulation of M-CAM expression in C2C12 cell line by RNAi. (A,B) Negative control (no M-CAM knockdown). (C,D) M-CAM oligonucleotide 20. (E,F) M-CAM oligonucleotide 38. (A,C,E) Immunofluorescence images of each culture stained for M-CAM expression. Note the reduction of M-CAM expression in C and E compared with A. (B,D,F) Cultures at day 7 after addition of differentiation medium stained for expression of myosin heavy chain (green) and with DAPI (blue) to visualize the nuclei. Magnification, 400× (A,C,E); 100× (B,D,F). (G) Quantitative real-time PCR of M-CAM RNA in control, oligo 20- and oligo 38-treated C2C12 cultures. Cultures treated with oligo 20 yielded a 60% reduction in M-CAM mRNA expression, whereas cultures treated with oligo 38 yielded an 87% reduction of M-CAM mRNA. (H) Fusion

index determined for each culture after differentiation was induced for 3, 7 and 12 days. The fusion index was calculated as the number of nuclei within myotubes divided by the total number of nuclei.

Table 1

Human fetal samples analyzed and percentage of myogenic cells

Sample	Age (weeks)	Desmin*	Early myotubes†	Late myotubes†
1	14	74%	2	4
2	17	90%	2	4
3	14	71%	1	2
4	13	70%	2	4
5	14	76%	2	3
6	18	88%	2	3
7	18	90%	2	3

* Percentage of desmin-positive cells detected in each sample. Desmin staining was performed on cells maintained in proliferation medium ~3 days after explantation.

† Number of days after addition of differentiation medium (day 0), at which total RNA was harvested for microarray analyses.