Journal of Cell Science 108, 2973-2981 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Involvement of M-cadherin in terminal differentiation of skeletal muscle cells

Michael Zeschnigk*, Detlef Kozian†, Christine Kuch, Marion Schmoll and Anna Starzinski-Powitz‡

Institut der Anthropologie und Humangenetik für Biologen, Johann-Wolfgang-Goethe-Universität, Siesmayerstrasse 70, D-60054 Frankfurt am Main, FRG

*Present address: Institut für Genetik der Universität zu Köln, Weyertal 121, D-50931 Köln, FRG
†Present address: Zellbiologie; Frauenklinik und Hebammenlehranstalt Georg-August-Universität Göttingen, Zentrum 11, Postfach 3742/43, D-37075 Göttingen, FRG
‡Author for correspondence

SUMMARY

Cadherins are a gene family encoding calcium-dependent cell adhesion proteins which are thought to act in the establishment and maintenance of tissue organization. M-cadherin, one member of the family, has been found in myogenic cells of somitic origin during embryogenesis and in the adult. These findings have suggested that M-cadherin is involved in the regulation of morphogenesis of skeletal muscle cells. Therefore, we investigated the function of M-cadherin in the fusion of myoblasts into myotubes (terminal differentiation) in cell culture. Furthermore, we tested whether M-cadherin might influence (a) the expression of troponin T, a typical marker of biochemical differentiation of skeletal muscle cells, and (b) withdrawal of myoblasts from the cell cycle (called terminal commitment). The studies were performed by using antagonistic peptides which correspond to sequences of the putative M-cadherin binding domain. Analogous peptides of N-cadherin have previously been shown to interfere functionally with the N-cadherin-mediated cell adhesion. In the presence of antagonistic M-cadherin peptides, the fusion of myoblasts into myotubes was inhibited. Analysis of troponin T revealed that it was downregulated at the protein level although its mRNA was still detectable. In addition, withdrawal from the cell cycle typical for terminal commitment of muscle cells was not complete in fusion-blocked myogenic cells. Finally, expression of M-cadherin antisense RNA reducing the expression of the endogenous M-cadherin protein interfered with the fusion process of myoblasts. Our data imply that M-cadherin-mediated myoblast interaction plays an important role in terminal differentiation of skeletal muscle cells.

Key words: morphogenesis, antagonistic peptide, antisense RNA, troponin T, DNA synthesis

INTRODUCTION

Vertebrate skeletal muscle cells arise from mesodermal stem cells which subsequently differentiate into determined myogenic precursor cells called myoblasts. During myogenesis, myoblasts withdraw from the cell cycle (terminal commitment), fuse into multinucleated myotubes (terminal differentiation) and express muscle specific proteins (biochemical differentiation) (Nadal-Ginard, 1978; Endo and Nadal-Ginard, 1987; Stockdale, 1992; Wachtler and Christ, 1992). Inter-myoblast recognition, alignment and fusion itself are characteristic steps of the fusion process. These steps depend on cell-cell as well as on cell-extracellular matrix interactions which are regulated, at least in part, by a variety of cell adhesion molecules (Knudsen, 1990; Dickson et al., 1990; Mège et al., 1992; Rosen et al., 1992).

One type of molecule likely to be involved in the fusion process are cadherins which constitute a multigene family. Cadherins are calcium dependent intercellular adhesion molecules and are thought to have morphoregulatory roles (Takeichi, 1988, 1991). In terminally differentiating mammalian skeletal muscle cells, two members of the cadherin family, M-cadherin and N-cadherin, have been identified. M-cadherin is already present in myoblasts and is upregulated after induction of myotube formation in culture (Donalies et al., 1991; Rose et al., 1994). Prenatally, it is only expressed in cells of the myogenic lineage (Moore and Walsh, 1993; Rose et al., 1994). In normal adult muscle, M-cadherin is present on satellite cells and most likely also on the sarcolemma underneath the satellite cells (Irintchev et al., 1994; Bornemann and Schmalbruch, 1994). During skeletal muscle regeneration, M-cadherin is up-regulated and can be identified on the regenerating myogenic structures (Moore and Walsh, 1993; Bornemann et al., 1994; Irintchev et al., 1994). All these findings imply a specific function for M-cadherin during muscle development and morphogenesis.

N-cadherin is also expressed in developing muscle cells (Hahn and Covault, 1992; Cifuentes-Diaz et al., 1994). In cell culture, inhibition of N-cadherin function by antibodies or synthetic peptides leads to a reduction but not to a complete block of chicken myoblast fusion (Knudsen et al., 1990a; Mège et al., 1992).

In order to investigate the role of M-cadherin during muscle formation, we aimed to block M-cadherin-mediated cell adhesion by adding antagonistic peptides to differentiation-induced rat L6 muscle cell cultures. The peptides used corre-

spond to extracellular M-cadherin sequences presumed to be involved in M-cadherin-binding since they are located at analogous positions to the binding sites of E-cadherin, N-cadherin and P-cadherin (Nose et al., 1990). In addition, L6 myoblasts which express RNA antisense to the 5′ end of M-cadherin mRNA were generated. Subsequently, such cells were tested for their ability to form myotubes. The results of both experimental approaches imply that M-cadherin plays an important role in terminal differentiation of skeletal muscle cells.

MATERIALS AND METHODS

Cell culture

Rat L6 myoblasts (Yaffe, 1968; re-cloned in our lab) were grown in Waymouth's medium containing 15% fetal calf serum and antibiotics. Formation of myotubes was induced at 80% confluency by switching cells to Dulbecco's modified Eagle's medium (DMEM) supplemented with insulin (10 μg/ml). The fusion index was used as a measure of morphological differentiation and was calculated by dividing the number of nuclei present in myotubes by the total number of nuclei counted. Approximately one thousand nuclei were counted per Giemsa-stained L6 cell culture. To test bromodeoxyuridine (BrdU) incorporation, cells were grown on calf skin collagen-coated (Sigma) coverslips. BrdU (10 μg/ml; Boehringer Mannheim) was added to L6 cells 36 hours after induction of differentiation. Twelve hours later, the cells were fixed and stained with BrdU antibodies according to the supplier's instructions (DAKO).

Synthetic peptides

The sequences of the synthetic peptides 040/M (putative binding region) and D559/M (N-terminal putative non-binding region) were deduced from the M-cadherin cDNA sequence and peptides 041/N (binding region) and D558/N (N-terminal non-binding region) from the mouse N-cadherin sequences (Miyatani et al., 1989; Nose et al., 1990). The amino acid sequences of all peptides are given in Table 1. Peptides were synthesized on an automatic peptide synthesizer (Milligene), purified by HPLC and sequenced. The peptides were solubilized in 0.1 M NaOH (peptides D559/M, 040/M and 041/N) or 0.15 N NaOH (peptide D558/N) at a concentration of 30-40 mg/ml, neutralized and diluted in DMEM to the concentrations indicated before being added to cell cultures.

RNA and DNA techniques used in this work were performed according to standard procedures (Sambrook et al., 1989).

Expression vectors

M-cadherin cDNA fragment (bp 1-389; Donalies et al., 1991) was cloned into the *Bgl*II site of vector pHD (Müller et al., 1988) to allow expression of M-cadherin sequences either in sense (p-sense/Mcad) or antisense (p-antisense/Mcad) orientation. L6 myoblasts were cotransfected with CsCl-purified plasmid DNA of either p-antisense/Mcad or p-sense/Mcad, and pSV2neo (Southern and Berg, 1982), containing the G418/neomycin resistant gene, at a ratio of 50:1. Transformants were selected with G418 and subsequently treated as outlined in Fig. 7.

Western blot analysis

Protein extracts were separated in a reducing 10% SDS-polyacrylamide gel (120 μg protein/lane if not indicated otherwise), blotted onto membrane (Biometra) and visualized by staining with Ponceau dye to indicate equal loading of protein (Sambrook et al., 1989). After destaining, blots were incubated with troponin T antibody (Amersham) visualized with an alkaline phosphatase-conjugated secondary antibody (Dianova) using standard protocols (Sambrook et al., 1989). For analysis of M-cadherin, western blots were prepared accordingly and probed with affinity-purified rabbit anti-M-cadherin antibody. This antibody has been described elsewhere (Rose et al., 1994).

Table 1. Aminoacid sequences of synthetic Mcad and Ncad peptides

Mcad: (040)	RLRAFALDLGGSTLED
	¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦ ¦
Ncad: (041)	HLRAHAVDINGNQVEN
Mcad: (D559)	FSIDRFTGRVYLNAT
Ncad: (D558)	PINLPENSRGPFPQELVRIR

¦ = identical aminoacid
⁞ = conservative aminoacid exchange

RESULTS

Expression analysis of M-cadherin in myogenic rat L6 cells

Previous studies have shown that mammalian myogenic cells commonly express two cadherins, M- and N-cadherin, both in situ as well as in cell culture (Hahn and Covault, 1992; Knudsen et al., 1990a; Pouliot et al., 1994). Since we wanted to analyze the role of M-cadherin without the functional interference of other members of the cadherin family, we chose the L6 cell myogenic cell line for our studies in which only a single cadherin, M-cadherin, has been identified (Pouliot et al., 1990, 1994, and unpublished results).

The expression of M-cadherin in L6 cells was examined on western blots before and at different times after induction of differentiation. The results of this experiment indicated that L6 myoblasts (Fig. 1, lane 1) as well as differentiating L6 cells (Fig. 1, lanes 2-4) express M-cadherin and that the expression is upregulated after induction of differentiation. This is in agreement with the analysis of M-cadherin in myogenic mouse C2C12 cells (Donalies et al., 1991; C. Kuch et al., unpublished data) and with recently published analyses of M-cadherin in L6 cells (Pouliot et al., 1994). Frequently, we observed staining of a double band with the M-cadherin antibodies in L6 myoblasts but not in differentiating L6 cells. We could not correlate the lower molecular mass band to an additional M-cadherin isoform and it might therefore correspond to a degradation product.

Inhibition of myotube formation with synthetic M-cadherin peptides

In order to block the function of M-cadherin, a synthetic 16mer peptide (040/M) derived from the putative ligand binding site was synthesized. This peptide was chosen by analogy to the homophilic binding regions known for E-, N- and P-cadherin (Nose et al., 1990). A synthetic peptide (041/N) was also deduced from the binding region of N-cadherin. A similar peptide has been shown to block the N-cadherin dependent outgrowth of nerves (Doherty et al., 1991) and to decrease the fusion of avian myoblasts into myotubes (Mège et al., 1992). In addition, control peptides from the most N-terminal regions of M-cadherin (D-559/M) and N-cadherin (D558/N) were prepared. All peptides are listed in Table 1. Peptides were added to L6 cell cultures when they were shifted to fusion

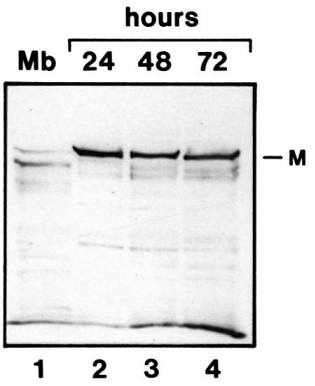

Fig. 1. Western blot analysis of M-cadherin protein with polyclonal M-cadherin antibodies in L6 cells at 24, 48 and 72 hours after induction of myotube formation (lanes 2-4) and in L6 myoblasts (lane 1). M-cadherin (labelled with M; runs at approximately 130 kilodalton; Rose et al., 1994) was detected using the ECL system (Amersham). Each lane contains 200 μg of cell extract (see also Material and Methods). Prior to labelling with M-cadherin antibodies the membrane was stained with Ponceau dye in order to indicate equal loading of protein (not shown). The double band in L6 myoblasts is regularly seen in western blots with myoblasts. Since no M-cadherin mRNA variant could be attributed to this second band (unpublished data), it might correspond to a degradation product.

Table 2. Fusion indices* (FI) and standard deviations (s.d.) of peptide-treated L6 cell cultures shown in Fig. 2

Peptide	Peptide concentration μg/ml	FI	s.d.
No peptide		0.874	0.038
040/M	400	0.028	0.025
	200	0.363	0.041
	100	0.597	0.068
	50	0.743	0.061
041/N	400	0.541	0.061
	200	0.714	0.036
	100	0.802	0.019
	50	0.835	0.037
D559/M	400	0.823	0.046
	200	0.824	0.016
	100	0.812	0.054
	50	0.809	0.022
D558/N	400	0.823	0.004
	200	0.775	0.039
	100	0.867	0.027
	50	0.832	0.075

*Fusion index: $\frac{\text{number of nuclei in myotubes}}{\text{number of total nuclei}}$

(Approximately 1,000 nuclei were counted per culture)

medium and not renewed during the remaining culture period. Myotube formation indicated by the fusion index was determined four days after induction of differentiation when maximal fusion was reached in control cultures.

Addition of M-cadherin peptide 040/M to L6 cell cultures induced to terminal differentiation reduced their capacity to form myotubes in a dose-dependent manner from 87% (no peptide; Figs 2 and 5B; Table 2) to 3% (400 μg/ml of peptide; Figs 2 and 5D; Table 2). Peptide D559/M, from the more N-terminal portion of M-cadherin, had no effect on myotube

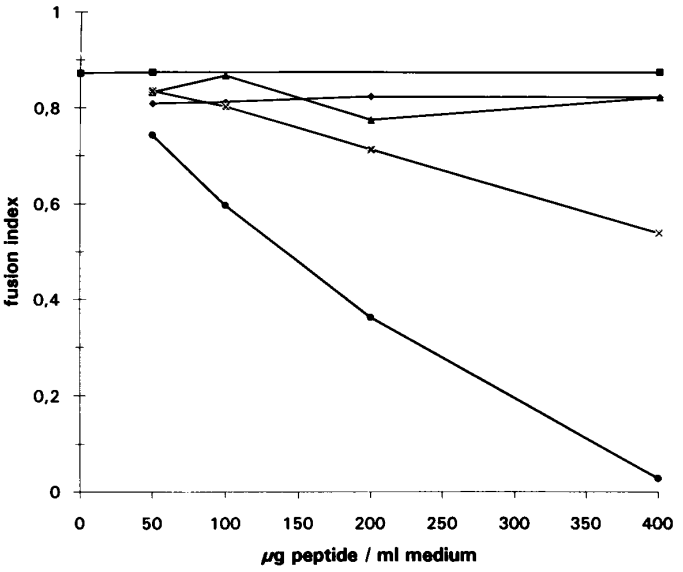

Fig. 2. Inhibition of myotube-formation of L6 myoblasts by synthetic M-cadherin peptides. Fusion indices of L6 cells in the absence (control, squares) or in the presence of different concentrations of synthetic M-cadherin peptides 040/M (putative binding region, circles) and D559/M (N-terminal region, diamonds) or N-cadherin peptides 041/N (binding region, crosses) and D558/N (N-terminal region, triangles) are shown. Peptides were added once to the L6 cell cultures when cells were shifted to differentiation medium. Cells were stained with Giemsa at day four after induction of myotube formation and the fusion indices were determined. Each fusion index represents the mean value of three independent cultures from each of which approximately 1,000 nuclei were counted. The exact fusion indices and standard deviations are given in Table 2. The fusion index is the number of nuclei in myotubes divided by the total number of nuclei counted.

formation (Figs 2 and 5C; Table 2). This indicated that peptide 040/M competed the binding of endogenous M-cadherin to its ligand and thus, is most likely part of the ligand-binding region of M-cadherin.

In the same series of experiments, increasing concentrations of peptide 041/N from the N-cadherin binding region were also added to differentiation-induced L6 cells. Since L6 cells do not express N-cadherin (our unpublished observations; Pouliot et al., 1994) peptide 041/N should exhibit either no or only partial inhibition of fusion. The latter might be possible because peptides 040/M and 041/N correspond to functionally analogous sequences and are rather similar. They share seven out of sixteen amino acid residues and five of the exchanges are conservative (Table 1). As can be seen in Fig. 2, peptide 041/N inhibited myotube formation. However, the inhibition was considerably less effective than the inhibition observed with peptide 040/M. Fusion ranged from 87% (no peptide and control peptide) to 54% (400 μg/ml of peptide). Again, control peptide D558/N from the N-terminal part of N-cadherin did not inhibit myotube formation. Most likely, N-cadherin peptide 041/N acts by cross-reaction with M-cadherin or its ligand in L6 cells.

Analysis of troponin T expression and DNA synthesis in M-cadherin-blocked cells

Subsequently, we asked whether M-cadherin might also

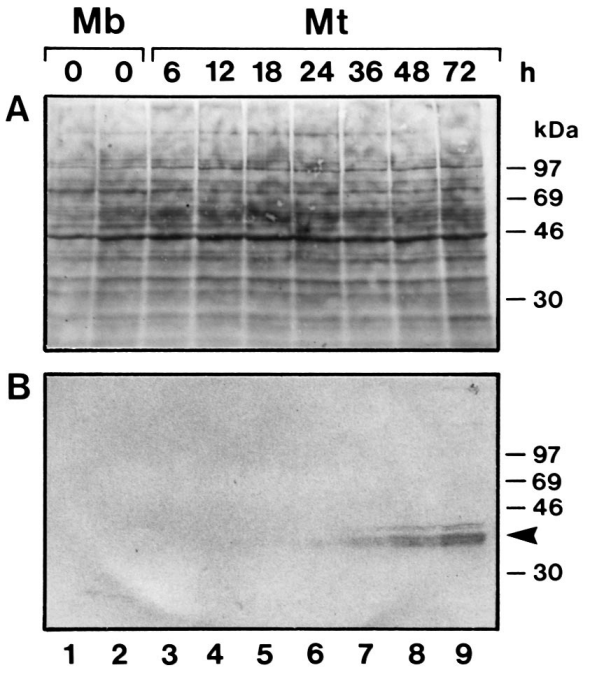

Fig. 3. Western blot analysis of troponin T protein in differentiating L6 cells at various times (h, hours) after induction of myotube formation (Mt; lanes 3-9) as well as in growing (lane 1) and confluent (lane 2) L6 myoblasts (Mb). In order to indicate equivalent loading of protein onto the gel, the membrane was first stained with Ponceau dye (A) and then probed with troponin T antibody (B). Molecular mass markers are indicated in kilodaltons (kDa).

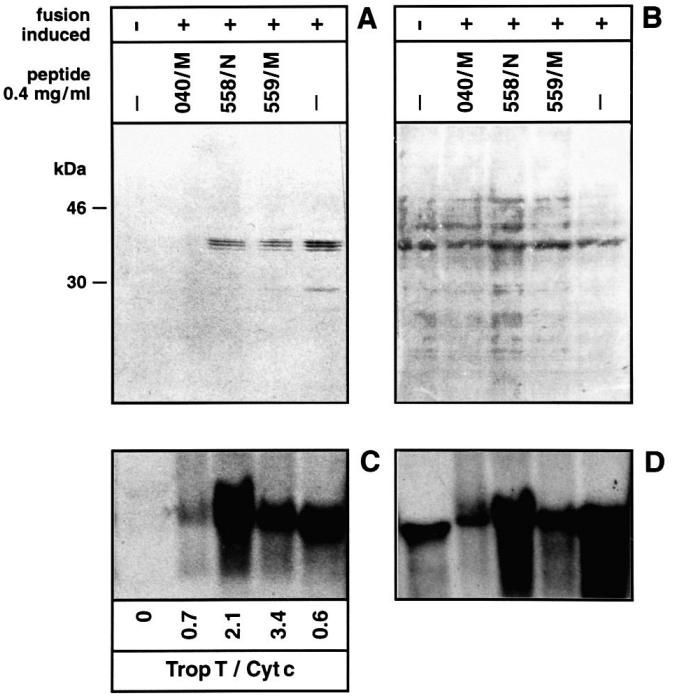

Fig. 4. Analysis of troponin T protein (A) and mRNA (C) in L6 myoblasts (–,–) and in differentiation-induced L6 cell cultures containing 400 μg/ml of either blocking M-cadherin peptide 040/M, control peptides D559/M from M-cadherin and D558/N from N-cadherin or no addition (–,+) four days after induction of myotube formation. (A) Western blot analysis of troponin T protein with a monoclonal troponin T antibody. (B) Ponceau-staining of the western blot membrane prior to incubation with troponin T antibody to show equivalent loading of samples. (C) Northern blot with total cytoplasmic RNA isolated from L6 cells which were treated as described for part (A) of this figure and probed with ^{32}P-labelled troponin T cDNA (Donalies et al., 1991). The membrane was re-probed with cytochrome *c* oxidase cDNA (Donalies et al., 1991) to indicate the integrity of the RNA samples tested. Scanning analysis of the northern blot autoradiographies was performed with a Joyce Loebl Chromoscan 3 instrument in absorbance mode. Aperture width = 0.5 mm. The integrals of the peaks were measured by the instrument. The ratio for each RNA sample is the quotient of the integral of the troponin T band and the integral of its corresponding cytochrome *c* oxidase band.

influence other parameters typical for differentiation of skeletal muscle cells. Therefore we analyzed the expression of a muscle specific protein, troponin T (Härtner et al., 1989; Wade et al., 1990) which is turned on during myotube formation in L6 cells and is therefore a biochemical marker of myogenic differentiation (Fig. 3). Furthermore we examined withdrawal of L6 cells from the cell cycle. Characteristically, myoblasts become post-mitotic prior to fusion both in vivo and in cell culture (Wachtler and Christ, 1992; Nadal-Ginard, 1978).

To evaluate the influence of M-cadherin on troponin T expression, fusion of L6 cells was blocked with peptide 040/M or treated with control peptides 558/N or 559/M to allow myotube formation. Analysis of troponin T was performed four days after induction of myotube formation (Fig. 4) since untreated L6 cells showed high expression of troponin T three days after differentiation (Fig. 3). As demonstrated by western blot experiments (Fig. 4A), differentiation-induced L6 cells did not contain any detectable troponin T protein in the presence of the blocking peptide derived from M-cadherin. In contrast, L6 cells incubated with control peptides showed expression of troponin T at levels comparable to those of untreated cultures.

From the same set of cultures, cytoplasmic RNA was prepared in order to analyze expression of troponin T mRNA. Northern blots hybridized with the troponin T probe (Fig. 4C) showed that troponin T mRNA was absent in L6 myoblasts (negative control) and expressed in differentiation-induced L6 cells which were cultured without any peptide or with control peptides (positive controls). Differentiation-induced L6 cells treated with the blocking peptide 040/M still showed detectable levels of troponin T mRNA. Comparison of the hybridization signals obtained with the troponin T probe (Fig. 4C) with the corresponding signals of the cytochrome *c* oxidase probe (Fig. 4D) by scanning analysis suggested that the troponin T signal from fusion-blocked cells was similar to those of control cultures.

From these analyses, we concluded that inhibition of M-cadherin by antagonistic peptides affected the accumulation of troponin T protein but not the activation of its gene.

As can be seen by comparison of Fig. 5A and D, the cell densities were higher in fusion-blocked L6 cell cultures than in non-induced L6 myoblast cultures which were kept in growth medium. This suggested that cell division was still occurring in differentiation-induced L6 cell cultures which were incubated with blocking peptide 040/M. In order to evaluate this observation, the incorporation of BrdU was tested at 36 hours after the induction of differentiation (Fig. 6). At

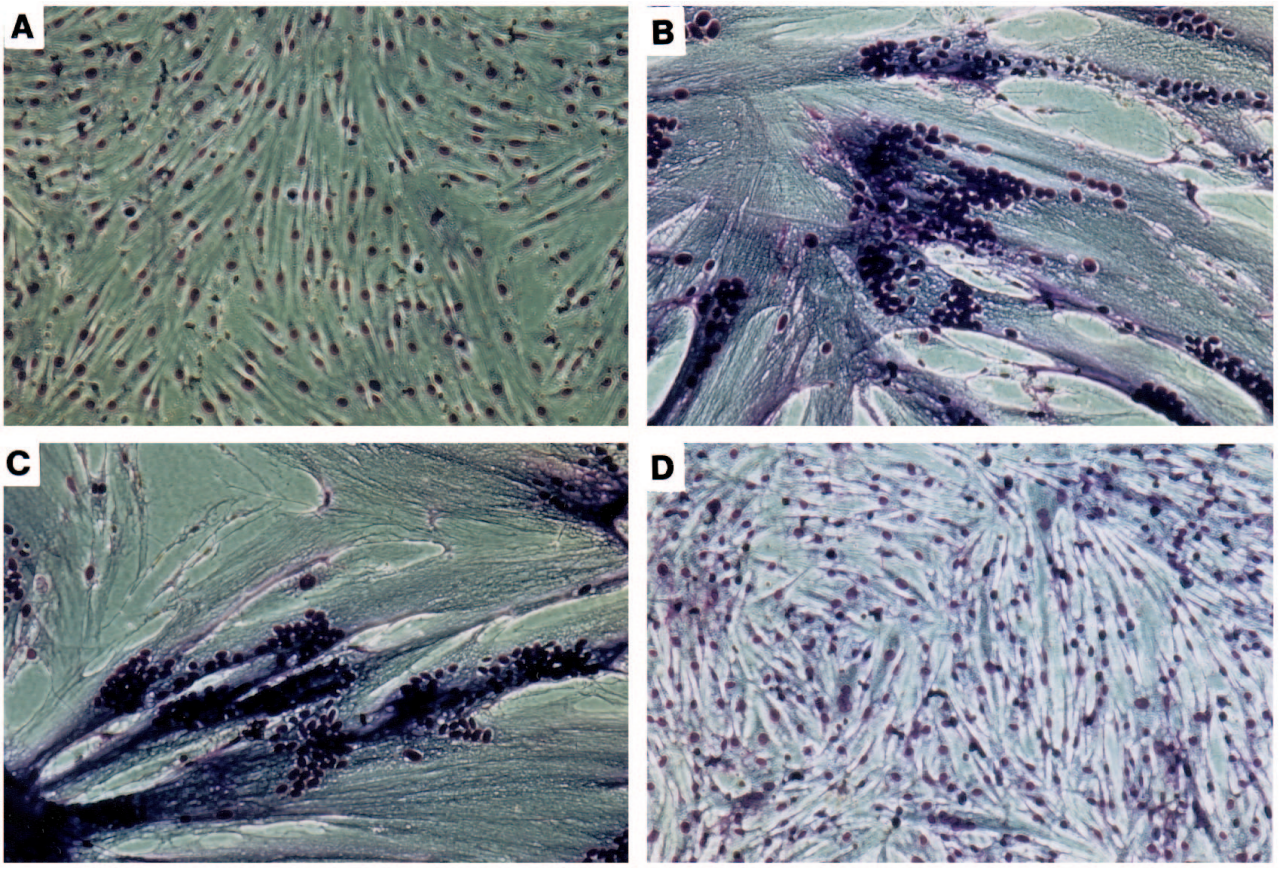

Fig. 5. Inhibition of myotube-formation of L6 myoblasts by synthetic M-cadherin peptides. (A) Confluent L6 myoblasts kept in growth medium instead of differentiation medium. (B) Myotube-formation of L6 cells in the absence of peptide or (C) in the presence of control peptide D559/M (400 μg/ml). (D) L6 myoblasts induced to myotube-formation in the presence of peptide 040/M (400 μg/ml). L6 myoblasts were induced to form myotubes by shifting them to differentiation medium (B-D). Peptides were added once when the cells were shifted to differentiation medium. Cultures were stained with Giemsa four days after induction of differentiation.

this time, differentiation-induced L6 cells have usually withdrawn from the cell cycle. In agreement with this expectation, differentiation-induced L6 cells without blocking peptide showed only background incorporation of BrdU. However, L6 cells induced to differentiation in the presence of the blocking peptide 040/M showed enhanced BrdU-incorporation. The highest number of BrdU-positive cells that we observed in the fusion-blocked L6 cell cultures was approximately 30% (Fig. 6) although this number could vary in independent experiments. Control experiments were also performed with confluent (negative control) and with exponentially growing (positive control) L6 myoblasts. Confluent L6 myoblasts did not show incorporation of BrdU whereas 80-90% of the exponentially growing L6 myoblasts were intensely labelled.

Thus, the incorporation of BrdU in fusion-blocked L6 cells supported the morphological observation that at least a portion of these cells were still able to divide. Finally, there was no indication of cell death in the fusion-blocked L6 cells implying that inhibition of myotube formation by antagonistic M-cadherin peptides is not caused by cell loss.

Transfection of L6 cells with M-cadherin antisense constructs

To analyze the function of M-cadherin by an independent experimental approach we aimed at reducing the translation of M-cadherin protein in L6 cells by expression of an antisense RNA. It is important to note that during culture, L6 myoblasts can develop into variants which are unable to differentiate biochemically and to fuse into myotubes. This number is even increased when L6 cells are kept in the presence of the antibiotic G418. The molecular defect in the non-differentiating L6 cells might vary from clone to clone and is difficult to evaluate on the basis of individual L6 cell clones. Therefore, it was essential to do a statistical analysis by comparing the number of L6 cell clones with low or no fusion capacity containing the antisense construct with those containing the sense construct.

As outlined in Fig. 7A, a 5′ fragment of M-cadherin cDNA was cloned in sense (p-sense/Mcad) and antisense (p-antisense/Mcad) orientation into the mammalian expression vector pHD. L6 myoblasts were co-transfected with either of these constructs and with plasmid pSV2neo. Fifteen G418-resistant colonies per construct (primary colonies) were randomly isolated, expanded and each of them was replated at a density of 600 cells per 130 cm^2. Usually, approximately 200 clones were eventually detected after replating. When the clones had reached a size of about 1,000 cells, differentiation was induced. Four days later, the fusion indices of about fifty single clones derived from one primary colony were deter-

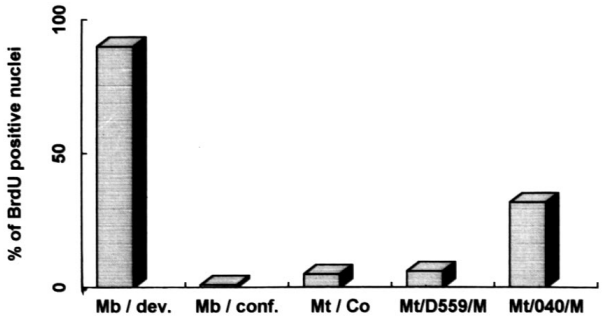

Fig. 6. Incorporation of BrdU into nuclei of L6 cells. BrdU was added to myoblasts 18 hours after plating (= growing myoblasts; Mb/dev.); to quiescent (confluent) myoblasts (Mb/conf.); to L6 cells 36 hours after induction of myotube formation without any addition (Mt/Co), in the presence of 400 μg/ml control peptide D559 (Mt/D559/M) or in the presence of 400 μg/ml M-cadherin peptide 040/M (Mt/040/M). Only growing myoblasts and myotube-induced cultures in the presence of fusion-blocking peptide 040/M showed intense immunofluorescence staining whereas the other cultures were negative.

mined. In this way, 984 clones of the p-antisense/Mcad transformants and 725 clones of the p-sense/Mcad transformants were analyzed in one experiment. A similar number of clones was analyzed in a second independent transfection experiment which revealed a comparable result like the one shown here.

The statistical analysis of the experiment shown in Fig. 7B demonstrated that expression of the M-cadherin antisense construct in L6 cells significantly reduced their potential to fuse. Analysis of the pooled data revealed that eighty-four percent of the p-antisense/Mcad cell clones showed reduced fusion capacity or no fusion at all as compared to fifty-five percent of the p-sense/Mcad clones. This difference was also reflected at the level of individual colonies (Table 3) although both, p-antisense/Mcad and p-sense/Mcad colonies contained non-fusing clones. However, the number of colonies with 70% or more non-fusing clones was higher in p-antisense/Mcad transformants than in p-sense/Mcad transformants (nine out of 15 as compared to 2 out of 14; see also Table 3). In addition, the p-sense/Mcad colonies exhibited a better overall fusion capacity than the p-antisense colonies.

In order to analyze whether expression of M-cadherin protein in the antisense clones correlated with the fusion potential of these cells, randomly selected p-sense/Mcad and p-antisense/Mcad myoblasts were tested for their expression of M-cadherin protein in western blot experiments using M-cadherin antibodies. Fig. 8 shows that the amount of M-cadherin protein corresponds to the fusion capacity of the respective colony. Colonies exhibiting low fusion express less M-cadherin than those showing intermediate or high fusion.

Thus, the data obtained with the antisense RNA approach are in agreement with the results of the experiments using the antagonistic M-cadherin peptides.

Table 3. Fusion indices (FI) of antisense (13.X) and sense clones (S.X) in individual primary colonies

	% Clones			
Colony no.	FI > 0.6	FI 0.2-0.6	FI < 0.2	FI 0
13.13	0	0	0	100
13.3	0	0	3	97
13.4	0	0	4	96
13.5	2	4	0	94
13.1	0	10	4	86
13.9	2	6	6	86
13.2	0	2	12	86
13.12	0	10	17	73
13.14	2	12	16	70
13.10	2	17	15	66
13.8	4	19	12	65
13.11	1	22	20	57
13.15	6	13	25	56
13.6	6	29	12	53
13.7	36	18	15	31
S.11	0	2	2	96
S.3	0	12	13	75
S.1	4	23	6	66
S.2	0	26	11	64
S.13	4	19	19	58
S.8	2	25	19	54
S.5	7	20	20	53
S.17	15	31	8	46
S.9	2	29	24	45
S.4	15	31	13	42
S.16	18	46	6	30
S.7	37	21	17	25
S.10	70	16	10	4
S.6	66	32	0	2
S.12	36	58	6	0

DISCUSSION

We have investigated the role of M-cadherin during the differentiation of skeletal muscle cells in culture. The results of two independent experimental approaches indicated that M-cadherin plays a crucial role in the terminal differentiation of myogenic cells. This idea is in accordance with the expression of M-cadherin in vivo: prenatal expression of M-cadherin is restricted to myogenic cells (Moore and Walsh, 1993; Rose et al., 1994). In the adult, M-cadherin is present on quiescent satellite cells, and is upregulated during skeletal muscle regeneration (Bornemann and Schmalbruch, 1994; Irintchev et al., 1994). In addition to skeletal muscle cells, postnatal expression of M-cadherin was also found in the cerebellum (Rose et al., 1995).

It is now well established that at least two cadherins, M- and N-cadherin, are expressed during the development of skeletal muscle (Cifuentes-Diaz et al., 1994; Rose et al., 1994). In cell culture, both cadherins have been suggested to be involved in the fusion of myoblasts to myotubes. Antagonistic peptides to N-cadherin do substantially, but not completely, inhibit fusion of avian myoblasts (Mège et al., 1992). No chicken homologue of mouse M-cadherin has been identified so far. In this study we show that antagonistic peptides to M-cadherin or expression of M-cadherin antisense RNA inhibited the fusion of L6 myoblasts. These cells express M-cadherin but not N-cadherin. Thus, the fusion process of myoblasts appears absolutely dependent on cadherin function but can be also regulated by M-cadherin in the absence of N-cadherin. It is possible, however, that both molecules contribute to the control of myoblast fusion in cells which jointly express M- and N-cadherin. This would be in line with a variety of recent experiments using gene targeting in the mouse which have demonstrated that important developmental events are generally regulated by multiple and functionally overlapping molecules.

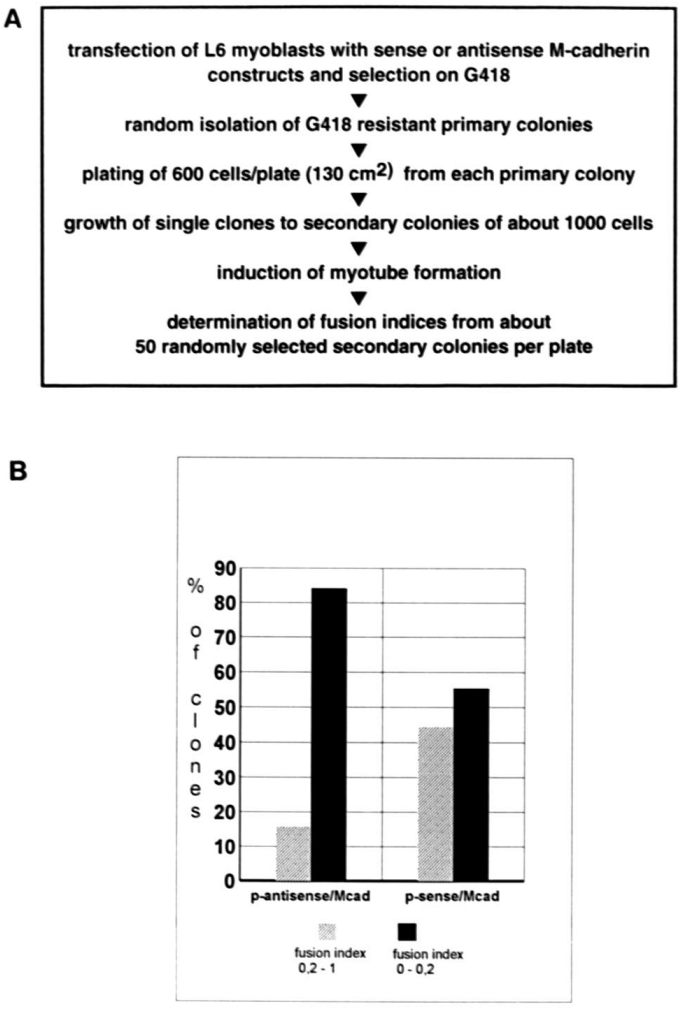

Fig. 7. Inhibition of M-cadherin by expression of M-cadherin antisense RNA. (A) Outline of the experimental design. (B) Percentage of G418-resistant L6 cell clones with reduced or no fusion potential (dark columns: fusion index 0-0.2) and normal fusion capacity (light shaded columns: fusion index 0.2-1) from randomly isolated colonies transfected with either p-antisense/Mcad (antisense construct;) or p-sense/Mcad (sense construct). *P*-values ($P \leq 0.001$; Student's *t*-test; Weber, 1986) indicate statistically significant differences in fusion properties between p-antisense/Mcad and p-sense/Mcad-transfected clones. Fusion indices were determined four days after induction of myotube formation.

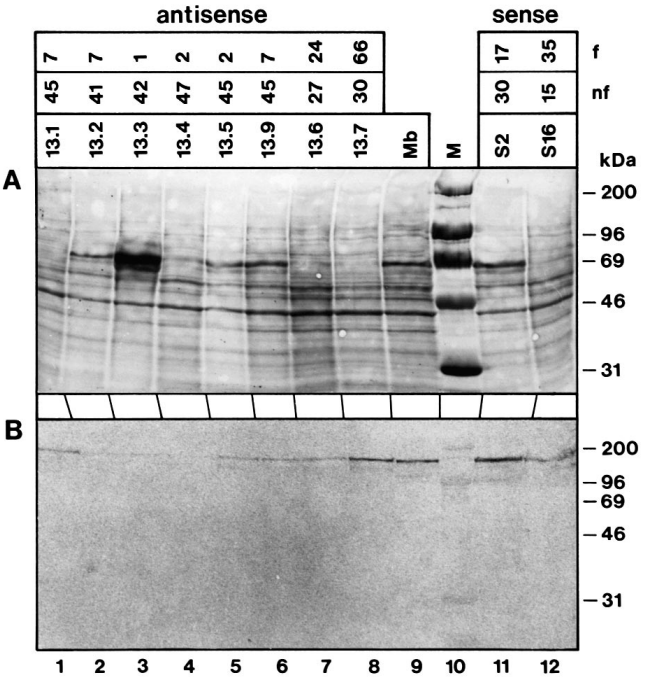

Fig. 8. Western blot analysis of M-cadherin in L6 cell colonies containing antisense (lanes 1-8) or sense constructs (lanes 11 and 12; see legend to Fig. 7). The line of (f) indicates the number of fusing clones of the particular primary colony tested and of (nf) the number of non-fusing clones. Mb: L6 cell myoblasts (lane 9). Sizes of protein markers (M; lane 10) are given in kilodaltons (kDa). In order to indicate equivalent loading of protein the membrane was stained with Ponceau dye (A) prior to probing with M-cadherin antibody (B).

Thus, N-cadherin might be redundant to the function of M-cadherin. Alternatively or, in addition, N-cadherin might control other aspects of myogenic morphogenesis than M-cadherin such as the establishment of the neuromuscular junction at which it has been identified in adult muscle (Cifuentes-Diaz et al., 1994).

As indicated by several studies, classical cadherins perform their function by homophilic intercellular binding (Miyatani et al., 1989; Nose et al., 1990; Takeichi, 1988, 1991). The N-terminal portion of the cadherins is essential for intercellular binding and provides the specificity of recognition of the respective cadherin (Nose et al., 1990). One particular peptide sequence necessary for interaction of the classical cadherins is centered around a tripeptide, the so-called HAV sequence (Nose et al., 1990). The HAV sequence is not present in M-cadherin which contains a FAL tripeptide instead (Donalies et al., 1991). Peptide 040/M containing the FAL motive has profound effects on myotube formation indicating that in M-cadherin the FAL tripeptide is not only located in an analogous position but also functions in a similar manner to the HAV sequence of the classical cadherins. Peptide 040/M most probably block homophilic interaction of M-cadherin. This is supported by our finding that $LMTK^-$ cells ectopically expressing M-cadherin do show aggregation (C. Kuch et al., unpublished results) in a fashion similar to E-cadherin and N-cadherin transfectants (Miyatani et al., 1989; Nose et al., 1990).

Besides being used in analysis of myogenesis, antagonistic peptides have generally been proven to be useful tools for the functional analysis of cadherins (Doherty et al., 1991; Mège et al., 1992). They obviously bind to cadherins but are unable to induce the signals required for the function of the cadherins. It seems that they simply occupy the binding region which would then be inaccessible for an intact cadherin. Finally, it seems unlikely that antagonistic M-cadherin peptides interfere with fusion by perturbing the membrane physiology since the functional importance of M-cadherin in the fusion of myoblasts was also demonstrated by a second independent approach, the expression of M-cadherin antisense RNA.

Troponin T protein is absent in M-cadherin-blocked L6 cells although its corresponding mRNA was still detectable. This result is reminiscent of previously reported experiments in

which L6 cells have been inhibited to fuse by EGTA. Such cells accumulated troponin T mRNA but not the corresponding protein (Endo and Nadal-Ginard, 1987). Conceivably, removal of calcium ions, usually required for cadherin-mediated adhesiveness (Ozawa et al., 1990), mimics the inhibitory effect of M-cadherin peptide 040/M. Nevertheless, removal of calcium ions from differentiation-induced L6 cells should have additional effects rather than simply inactivating M-cadherin. Therefore, down-regulation of troponin T might be achieved by different mechanisms in EGTA-treated and M-cadherin-blocked L6 cells, for example at the translational level in one case or an increased rate of degradation in the other case.

Troponin T is one of the contractile proteins in skeletal muscle cells which is physically associated with the myofilament network. The observation that troponin T was down-regulated at the protein level raises the question whether M-cadherin might influence (directly or indirectly) the formation or organization of myofilament elements to which troponin T has to be associated. Disturbance of such myofilament structures by inhibition of the M-cadherin function might prevent incorporation of troponin T finally leading to its rapid degradation. Support for the idea that M-cadherin might influence the formation or organization of myofilament structures comes from studies of N- and E-cadherin. In one of these studies, it has been shown that perturbance of N-cadherin in cardiomyocytes by N-cadherin antibodies decreased cell-cell contacts and also resulted in the disorganization of myofibrils (Soler and Knudsen, 1994). Furthermore, E-cadherin has been proposed to be associated with the actin-based cytoskeletal network (Hirano et al., 1987; Ozawa and Kemler, 1992; Kemler, 1993; Hinck et al., 1994). Current models favour the idea that E-cadherin is linked to the actin-based cytoskeleton via cytoplasmic proteins, the catenins, with a possible involvement of additional proteins such as vinculin and α-actinin (Kemler, 1993). It should be noted, however, that the evidence for an association of the cadherins with cytoskeletal or myofilament structures is still indirect.

Some of the L6 cells blocked in fusion by the M-cadherin peptide 040/M showed DNA synthesis as revealed by the incorporation of BrdU. This was in agreement with the observation that fusion-blocked L6 cell cultures appeared to contain more cells than confluent L6 myoblasts suggesting continuing cell division. One explanation for this phenomenon might be that peptide 040/M decreased the strength of M-cadherin-mediated intercellular adhesion which might be one of the intercellular signals required for withdrawal from the cell cycle and for myoblast fusion but not for biochemical differentiation. In line with this idea are the findings that M-cadherin is transiently upregulated during terminal differentiation of skeletal muscle cells (Pouliot et al., 1994; Rose et al., 1994; Irintchev et al., 1994; C. Kuch et al., unpublished data). But M-cadherin should perform its function, at least in part, in concert with other cell adhesion molecules which can also modulate the terminal differentiation of skeletal muscle cells. In addition to N-cadherin which we already discussed, such molecules might be N-CAM (Dickson et al., 1990; Knudsen et al., 1990b; Peck and Walsh, 1993) and integrins (Rosen et al., 1992).

We thank C. Birchmeier and G. Butler-Browne for critical reading of the manuscript, Y. Zimmermann for peptide synthesis, C. Siewert and S. Becker for technical assistance and U. Ringeisen and E. Kiessling for photographs. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 169.

REFERENCES

Bornemann, A. and Schmalbruch, H. (1994). Immunocytochemistry of M-cadherin in mature and regenerating rat muscle. *Anat. Rec.* **239**, 119-125.

Cifuentes-Diaz, C., Nicolet, M., Goudou, D., Rieger, F. and Mège, R. M. (1994). N-cadherin expression in developing, adult and denervated chicken neuromuscular system: accumulations at both the neuromuscular junction and the node of Ranvier. *Development* **120**, 1-11.

Dickson, G., Peck, D., Moore, S. E., Barton, C. H. and Walsh, F. S. (1990). Enhanced myogenesis in NCAM transfected mouse myoblasts. *Nature* **344**, 348-351.

Doherty, P., Rowett, L. H., Moore, S. E., Mann, D. and Walsh, F. S. (1991). Neurite outgrowth in response to transfected NCAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. *Neuron* **6**, 247-258.

Donalies, M., Cramer, M., Ringwald, M., and Starzinski-Powitz, A. (1991). Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. *Proc. Nat. Acad. Sci. USA* **88**, 8024-8028.

Endo, T. and Nadal-Ginard, B. (1987). Three types of muscle-specific gene expression in fusion-blocked rat skeletal muscle cells: translational control in EGTA-treated cells. *Cell* **49**, 515.

Hahn, C. G. and Covault, J. (1992). Neural regulation of N-cadherin gene expression in developing and adult skeletal muscle. *J. Neurosci.* **12**, 4677-4687.

Härtner, T., Kirschbaum, B. J. and Pette, D. (1989).The multiplicity of troponin T isoforms. Distribution in normal rabbit muscles and effects of chronic stimulation. *Eur. J. Biochem.* **179**, 31-38.

Hinck, L., Näthke, I. S., Papkoff, J. and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* **125**, 1327-1340.

Hirano, S., Nose, A., Hatta, K., Kawakami, A. and Takeichi, M. (1987). Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J. Cell Biol.* **105**, 2501-2510.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A. and Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated and regenerating mouse muscles. *Dev. Dynam.* **199**, 326-337.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321.

Knudsen, K. (1990). Cell adhesion molecules in myogenesis. *Curr. Opin. Cell Biol.* **2**, 902-906.

Knudsen, K. A., Myers, L. and McElwee, S. A. (1990a). A role for the Ca^{2+}-dependent adhesion molecule, N-cadherin, in myoblast interaction during myogenesis. *Exp. Cell Res.* **188**, 175-184.

Knudsen, K. A., McElwee, S. A. and Myers, L. (1990b). A role for neural cell adhesion molecule, NCAM, in myoblast interaction during myogenesis. *Dev. Biol.* **138**, 159-168.

McNeill, H., Ozawa, M., Kemler, R. and Nelson, W. J. (1990). Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell* **62**, 309-316.

Mège, R. M., Goudou, D., Diaz, C., Nicolet, M., Garcia, L., Geraud, G. and Rieger, F. (1992). N-cadherin and NCAM in myoblast fusion: Compared localisation and effect of blockade by peptides and antibodies. *J. Cell Sci.* **103**, 897-906.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunaga, M., Hatta, K. and Takeichi, M. (1989). Neural cadherin: role in selective cell-cell adhesion. *Science* **245**, 631-635.

Moore, R. and Walsh, F. S. (1993). The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle. *Development* **117**, 1409-1420.

Munro, S. and Pelham, H. R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907

Müller, G., Ruppert, S., Schmid, E. and Schütz, G. (1988). Functional analysis of alternatively spliced tyrosine transcripts. *EMBO J.* **7**, 2723-2730.

Nadal-Ginard, B. (1978). Commitment, fusion, and biochemical differentiation of a myogenic line in the absence of DNA synthesis. *Cell* **15**, 855-864.

Nose, A., Tsuji, K. and Takeichi, M. (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* **61**, 147-155.

Ozawa, M., Engel J. and Kemler, R. (1990). Single amino acid substitutions in one Ca^{2+} binding site of uvomorulin abolish the adhesive function. *Cell* **63**, 1033-1038.

Ozawa, M. and Kemler, R. (1992). Molecular organisation of the uvomorulin-catenin complex. *J. Cell Biol.* **116**, 989-996.

Peck, D. and Walsh, R. S. (1993). Differential effects of over-expressed neural cell adhesion isoforms on myoblast fusion. *J. Cell Biol.* **123**, 1587-1595.

Pouliot, Y., Holland, P. C. and Blaschuk, O. W. (1990). Developmental regulation of a cadherin during the differentiation of skeletal myoblasts. *Dev. Biol.* **141**, 292-298.

Pouliot, Y., Gravel, M. and Holland, P. C. (1994). Developmental regulation of M-cadherin in the terminal differentiation of skeletal myoblasts. *Dev. Dynam.* **200**, 305-312.

Rose, O., Rohwedel, J., Reinhardt, S., Bachmann, M., Cramer, M., Rotter, M., Wobus, A. and Starzinski-Powitz, A. (1994). Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cell in culture. *Dev. Dynam.* **201**, 245-259.

Rose, O., Grund, C., Reinhardt, S., Starzinski-Powitz, A. and Franke, W. W. (1995). Contactus adhaerens, a special type of plaque-bearing adhering junction containing M-cadherin, in the granule cell layer of the cerebellar glomerulus. *Proc. Nat. Acad. Sci. USA* (in press).

Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J. and Dean, D. C. (1992). Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. *Cell* **69**, 1107-1119.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.

Soler, A. P. and Knudsen, K. A. (1994). N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. *Dev. Biol.* **162**, 9-17.

Southern, P. J. and Berg, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**, 327-341.

Stockdale, F. E. (1992). Myogenic cell lineages. *Dev. Biol.* **154**, 284-298.

Takeichi, M. (1988). The cadherins: Cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**, 639-655.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Wachtler, F. and Christ, B. (1992). The basic embryology of skeletal muscle formation in vertebrates: the avian model. *Dev. Biol.* **3**, 217-227.

Wade, R., Sutherland, C., Gahlmann, R., Kedes, L., Hardeman, E. and Gunning, P. (1990). Regulation of contractile protein gene family mRNA pool sizes during myogenesis. *Dev. Biol.* **142**, 270-282.

Weber, E. (1986). *Grundriss der biologischen Statistik*. Gustav Fischer Verlag, Stuttgart.

Yaffe, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Nat. Acad. Sci. USA* **61**, 477-483.

(Received 19 December 1994 - Accepted 1 June 1995)